University of Alberta

The role of Proteinase-Activated Receptor-2 (PAR-2) in allergic sensitization and allergic asthma

By

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Experimental Medicine

Department of Medicine

Edmonton, Alberta
Spring 2008

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Your file Votre référence
ISBN: 978-0-494-45417-6
Our file Notre référence
ISBN: 978-0-494-45417-6

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Abstract

Allergic asthma is classified as a type I hypersensitivity reaction. Such reactions have a sensitization phase and an effector phase. Proteinases have been implicated in mediating both phases.

The reasons why only a limited number of inhaled antigens induce allergic sensitization remain unclear. One interesting common characteristic of many potent allergens is that they possess serine proteinase activity or are in particles rich in proteinases. Such allergens could mediate their effects in the airways through the Proteinase-activated Receptor-2 (PAR-2). To mimic allergens that can activate PAR-2 we administered a PAR-2 activating peptide (PAR-2AP) with OVA antigen intranasally to mice. These mice developed allergic reactions in the lung characterized by airway inflammation and airway hyperresponsiveness (AHR), while mice given ovalbumin (OVA) alone or with a control peptide (PAR-2CP) did not. Furthermore, T cells isolated from the spleens of mice receiving OVA with PAR-2AP proliferated vigorously when re-stimulated with OVA in the presence of antigen presenting cells (APC) *in vitro* and displayed a Th2 phenotype. T cells from mice given OVA alone or with PAR-2CP proliferated poorly and displayed a tolerogenic phenotype. TNF has been shown to have potent effects on dendritic cells (DCs) thus we investigated its role in our model of PAR-2 induced allergic sensitization using an anti-TNF neutralizing antibody (Ab). Administration of the TNF neutralizing Ab prior to administration of OVA with PAR-2AP blocked sensitization.

In the effector phase of asthma serine proteinases, such as mast cell tryptase, trypsin-like enzymes, and certain allergens are important in the pathogenesis of asthma. These proteinases can activate PAR-2. PAR-2 is up-regulated in the airways of asthmatics. The purpose of this study was to investigate PAR-2 activation in the airways during allergen challenge. PAR-2AP or PAR-2CP was administered alone or in conjunction with ovalbumin (OVA) intranasally to mice and AHR and airway inflammation were evaluated. When administered with OVA, PAR-2AP enhanced AHR and airway inflammation when compared to OVA administered alone or with PAR-2CP. The enhanced AHR persisted for 5 days while the enhancement to airway inflammation dissipated. Mice administered PAR-2AP alone during the 5 days following the final antigen challenge demonstrated an additional enhancement to airway inflammation when compared to the control animals.

Dedication

I dedicate this thesis to my parents Kenneth Elmer Ebeling and the late Bernice Francis Ebeling. You two have always been there for me and I could have never of gotten through it without you. You two have taught me so much. I do not think I will ever have any teachers/mentors greater than you. Mom I wish you could have been here for this accomplishment in my life. It will always seem hollow to me as I know how excited you were to see it. I miss you so much.

I also dedicate this thesis to my sisters Donna and Debbie who also have been there for me and their excitement for me has pulled me through.

Finally special mention goes to Christina Jovillar who has been with me throughout this time. Her strength has helped me through the bad times along with smile, laugh, and innocence that I love so much. God bless you my love.

Preface

In this thesis I present two publications (Chapters 3 and 4) under the theme of the role of proteinases in allergic sensitization and allergic asthma. Proteinases can mediate many effects in the airways one of these being activation of Proteinase-Activated Receptors (PARs). Of the four members of the PAR family I focus specifically on the role that PAR-2 plays in the development of allergic sensitization and during allergic inflammation.

Chapter 1 is introductory. I describe asthma and how it is classified as a type I hypersensitivity reaction. I will describe two phases in asthma development (sensitization and effector phases) and evidence that PAR-2 activation is important in both phases. One important point is that many potent allergens either possess or are inhaled in particles rich in proteinases. These allergens could activate PAR-2 and this activation could mediate allergic sensitization. Another point is that proteinases are integral in allergic inflammation (effector phase) and that it is likely that PAR-2 is important in this process as well. A conceptual model is synthesized that was used to generate the hypotheses that mediators released following PAR-2 activation mediate allergic sensitization and that during allergic inflammation these mediators cause the inflammation to worsen.

In chapter 2 inflammatory mediators released in the airways following PAR-2 activation were studied. PAR-2AP was used to intranasally (i.n.) activate PAR-2 to Balb/c mice. Following PAR-2 activation, mRNA and protein levels of the mediators were studied. We found one important mediator, TNF, was not only up-regulated at the mRNA level but increased protein levels were also present in the broncheoalveolar lavage (BAL) following activation. We also found that PAR-2 activation alone did not initiate either eosinophillic airway inflammation or AHR.

In chapter 3 the first of two publications is presented. We used an allergic prone Balb/c model where we administered OVA alone, OVA with PAR-2CP, or OVA and PAR-2AP. OVA given alone or with PAR-2CP induced mucosal immune tolerance that was characterized by the lack of AHR, airway inflammation, or OVA-specific IgE. Furthermore, spleenic T cells from these animals proliferated poorly when re-stimulated *in vitro* with OVA. These T cells also secreted high amounts of IL-10, indicative of $T_{reg}1$ or Th3 function. Mice administered OVA with PAR-2AP developed AHR, airway inflammation, and OVA-specific IgE indicating allergic sensitization. Spleenic T cells from these animals proliferated vigorously when re-stimulated *in vitro* with OVA and showed a Th2 cytokine profile. To test whether the PAR-2 mediated effects were specific to the Balb/c strain of mouse we tested C57/B6 mice as well and found the effects seen in the Balb/c strain. Along with the development of allergic disease in the lung, we showed that pulmonary dendritic cell (DC) trafficking was different in mice given PAR-2AP. As shown in chapter 2 TNF was released following PAR-2 activation. Using a TNF neutralizing Ab we showed that PAR-2 mediated sensitization through this mediator.

In chapter 4 the second publication is presented. We used a Balb/c model of allergic asthma to study the effects of enhanced PAR-2 activation during allergen challenge (effector phase of allergic lung disease). When mice were sensitized and challenged with OVA ± PAR-2CP they developed eosinophilic airway inflammation and AHR. Mice receiving OVA and PAR-2AP showed enhancement to both of these pathological conditions. The PAR-2 mediated enhancement to AHR persisted when mice were assessed 5 days later, while enhancement to airway inflammation dissipated by this time. To address whether further PAR-2 activation, without OVA antigen, mice administered PAR-2AP alone during the 5 days following the final antigen challenge showed a further enhancement to airway inflammation when compared to the control animals. Finally we show that PAR-2AP administered with allergen increased TNF and IL-5 mRNA in lung tissue and IL-13 and TNF in BAL fluid.

In the last chapter, I summarize the major findings of these studies and provide a conceptual model to explain our observations. I integrate these findings with relevant literature, discuss their relevance and outline future research directions essential to advance this field.

Acknowledgements

I would like to acknowledge the help that I have received from the Pulmonary Research Group. Your help and advice has made this thesis possible. Of the group I would especially like to thank Melanie Abel whose help has been vital in conducting my research. I would also like to thank Dr. Wole Odemuyiwa for the talks about my work and my life that have helped me along the way.

I would also like to thank my supervisory committee members Dr. Allan Murray, Dr. A. Dean Befus, and my supervisor Dr. Harissios Vliagoftis.

Table of Contents

Chapter 1: General Introduction……………………………………………………..1

- I. Asthma……………………………………………………………………1
- II. Hypersensitivity…………………………………………………………..2
- III. Type I Hypersensitivity…………………………………………………4
  - i. Atopy and Allergic Diseases……………………………………4
  - ii. Determinants of Type I Hypersensitivity……………………………..6
    - Genetics………………………………………….....6
    - Environmental determinants………………………………7
- IV. Phases in the Development of Type I Hypersensitivity………………….8
- IV. A. Phase 1: Sensitization Phase……………………………………………..8
  - i. Cells critical in the Sensitization Phase……………………………8
    - Dendritic cells………………………………………..8
    - CD4^{+} T cells……………………………………………9
    - B cells…………………………………………………...10
  - ii. Important mediators in the Sensitization Phase………………...11
    - IL-4……………………………………………………11
    - IL-13…………………………………………………...12
  - iii. Cellular mechanisms in the Sensitization Phase……..…………13
  - iv. Danger signals that lead to atopic sensitization……..…………..16
    - Lipopolysaccaride (LPS) and TLR-4 interaction…..16
    - GM-CSF expressed in the airways…………………17

v. Allergen biology...18

- Common characteristics of potent allergens.................18
- Proteinase-mediated pathway in the development of allergic disease...19

V. Proteinase-Activated Receptors (PARs)................................20

i. PAR activation...20

ii. PAR-2..23

- Expression of PAR-2 in the body.................................23
- Allergens – exogenous proteinases in allergic type I sensitization...23

VI. Phase 2 of Type I hypersensitivity: Effector Phase..............24

VI. a. Early phase of the effector phase...............................25

a. i. Cells critical in the early phase reaction of the effector phase.....25

- Mast cells..25

a. ii. Important mediators in the early phase reaction...............26

- TNF...26
- Histamine and Leukotrienes......................................27
- Proteinases..28

a. iii. Cellular mechanisms in the early phase reaction of Type I Hypersensitivity..28

VI. b. Late phase reaction of the effector phase......................32

b. i. Cells critical in the late phase reaction of the effector phase......32

- Basophils..32

- Eosinophils……………………………………………...32
- Neutrophils……………………………………………...33
- NKT cells…………………………………………………34

b. ii. Important mediators in the late phase reaction…………………35

- IL-3…………………………………………………….35
- GM-CSF………………………………………………...35

b. iii. Cellular mechanisms in the late phase reaction
of Type I Hypersensitivity…………………………………36

VII. Role of PAR-2 during the effector phase of type I reactions…………...39

i. Endogenous activators of PAR-2 during the effector phase……..39

- Mast cell tryptase………………………………………39
- Trypsin…………………………………………………..40
- Neutrophil proteinases…………………………………40

ii. Effects of PAR-2 activation on resident lung cells………………..41

- Epithelium……………………………………………...41
- Smooth muscle cells……………………………………42
- Endothelium/vasculature………………………………42

iii. Immune cells recruited to the airways during the effector phase..43

- T cells…………………………………………………...43
- Mast cells………………………………………………43
- Neutrophils……………………………………………...44

iv. Importance of PAR-2 in allergic inflammation in the airways…..44

v. Role of PAR-2 in atopic dermatitis………………………………..45

VIII. Respiratory mucosal immune tolerance.....46
  i. Cells involved in the development of respiratory mucosal immune tolerance.....47
    - conventional "modulated DCs".....47
    - Plasmacytoid Dendritic cells.....48
    - $CD4^+$ $T_{reg}1$ and Th3 cells.....48
  ii. Important cytokines in the development of mucosal immune tolerance.....49
    - IL-10.....49
  iii. Molecular mechanisms of mucosal immune tolerance.....49
IX. Studying the role of PAR-2 in allergic sensitization and allergic inflammation using *in vivo* mouse models.....52
  i. Role of PAR-2 in the development of allergic sensitization.....52
  ii. Role of PAR-2 in the effector phase of atopic/allergic asthma.....53
  iii. Strain of mouse to be used.....54
  iv. Mouse models and human asthma.....54
X. Hypothesis and conceptual models.....55
  i. Role of PAR-2 in the development of atopic/allergic sensitization..55
    - Hypothesis.....55
    - Methodology.....55
    - Conceptual model.....56
  ii. Role of PAR-2 in the effector phase of atopic/allergic asthma.....58
    - Hypothesis.....58

- Methodology....................58
- Conceptual model....................58

IX. References....................60

Chapter 2: Results section #1....................111

I. Introduction....................112

II. Methods....................113

III. Results....................117

IV. Discussion....................123

V. References....................125

Chapter 3: Results section #2....................128

I. Introduction....................129

II. Methods....................133

III. Results....................138

IV. Discussion....................165

V. References....................169

Chapter 4: Results section #3....................178

I. Introduction....................179

II. Methods....................181

III. Results....................185

IV. Discussion....................198

V. References....................202

Chapter 5: General Discussion and Conclusions....................209

I. Summary of the major findings and their relation to the original aims/hypothesis....209
II. Conceptual model Revisited....211
III. Results and their relation to the current literature....217
IV. Relevance and clinical significance of our findings....221
V. Future Directions....222
VI. References....226

List of Figures

Figure 1.1: Cellular mechanisms of the sensitization phase of Type I Hypersensitivity........15

Figure 1.2: Mechanisms of PAR activation........22

Figure 1.3: Early phase reaction of the effector phase of Type I Hypersensitivity...31

Figure 1.4: Late phase reaction of the effector phase of Type I Hypersensitivity....38

Figure 1.5: Cellular mechanisms of mucosal immune tolerance........51

Figure 1.6: OVA administered into the airways promotes mucosal immune tolerance........57

Figure 1.7: Conceptual model: PAR-2 activation interferes with the development of mucosal immune tolerance and promotes allergic sensitization........57

Figure 1.8: Conceptual model: PAR-2 activation of specific cell types during allergen challenge enhances pathological features of asthma........59

Figure 2.1: PAR-2 localization in/on the lung tissue, alveolar macrophage, and nasal tissue of Balb/c mice........117

Figure 2.2: RNase protection assay for inflammatory cytokines present in the lung 4 h following administration of PAR-2CP or PAR-2AP........119

Figure 2.3: IL-13 and TNF levels in BAL fluid of sensitized mice treated with PAR-2CP or PAR-2AP........120

Figure 2.4: Total cell numbers in BAL as well as Penh values, from increasing doses of MeCh, in mice administered PAR-2AP or PAR-2CP alone........121

Figure 3.1: Protocols used to study the role of PAR-2 in the development of allergic sensitization...139

Figure 3.2: PAR-2 activation concurrently with mucosal exposure to OVA promotes AHR and airway inflammation...140

Figure 3.3: PAR-2 activation concurrently with mucosal exposure to OVA induces OVA-specific T cells that proliferate when re-stimulated with OVA in vitro...142

Figure 3.4: PAR-2 activation concurrently with mucosal exposure to OVA induces allergic sensitization...143

Figure 3.5: Memory response to OVA following PAR-2 activation concurrently with mucosal exposure to OVA...145

Figure 3.6: Measurement of OVA-specific immunoglobulins following PAR-2 activation concurrently with mucosal exposure to OVA.............................146

Figure 3.7: PAR-2AP administration with OVA prevents intranasal OVA induced mucosal airway tolerance...148

Figure 3.8: PAR-2 activation prevents mucosal airway tolerance to OVA..........150

Figure 3.9: PAR-2 activation prevents mucosal airway tolerance to OVA..........151

Figure 3.10: PAR-2 activation concurrently with mucosal exposure to OVA induces sensitization..153

Figure 3.11: PAR-2 activation with mucosal exposure to OVA induces sensitization in C57/B6 mice...155

Figure 3.12: DC antigen uptake and trafficking 6 h following PAR-2 activation...157

Figure 3.13: DC antigen uptake and trafficking 12 h following PAR-2 activation……158

Figure 3.14: DC antigen uptake and trafficking 24 h following PAR-2 activation……159

Figure 3.15: DC antigen uptake and trafficking 48 h following PAR-2 activation……160

Figure 3.16: DC antigen uptake and trafficking 72h following PAR-2 activation……161

Figure 3.17: PAR-2 induced allergic sensitization is TNF dependent……163

Figure 3.18: PAR-2 induced allergic sensitization is TNF dependent……164

Figure 4.1: OVA sensitization/challenge protocols used to study the role of PAR-2 on airway inflammation and AHR……186

Figure 4.2: BAL and AHR in mice subject to the protocols in Fig. 4.1……187

Figure 4.3: Flow cytometric analysis of BAL……188

Figure 4.4: Lung inflammation in ovalbumin-sensitized mice challenged with OVA and PAR-2CP or OVA and PAR-2AP……190

Figure 4.5: PAR-2 localization on inflammatory cells……193

Figure 4.6: RNase protection assay for inflammatory cytokines……195

Figure 4.7: TNF and IL-13 levels in BAL fluid of challenged mice……197

Figure 5.1: PAR-2 activation interferes with the development of mucosal immune tolerance and promotes allergic sensitization through a TNF mediated pathway….213

Figure 5.2: OVA sensitized mice challenged with OVA with PAR-2CP show a cuffing of eosinophils and neutrophils around the airway epithelium and endothelium..215

Figure 5.3: Conceptual model of the effects of PAR-2 activation during OVA challenge..216

List of Tables

Table 4.1: Serum levels of OVA-specific IgE in the short-term protocol.........191

List of Abbreviations

AHR: airway hyperresponsiveness

AM: alveolar macrophage

BCR: B cell receptor

DC: dendritic cell

FL-OVA: flourescent OVA

HDM: house dust mite

iDC: immature DC

Ig: immunoglobulin

IgA: immunoglobulin-A

IgE: immunoglobulin-E

IgG: immunoglobulin-G

IL-: interleukin

LPS: lipopolysaccaride

MHC: major histocompatibility complex

moDC: modulated DC

NKT: natural killer T cell

OVA: ovalbumin

PAR: Proteinase-Activated receptor

PAR-2CP: PAR-2 control peptide

PAR-2AP: PAR-2 activating peptide

pDC: plasmacytoid DC

TCR: T cell receptor

Th: T-helper cell

Th0: T-null cell (naïve T cell)

Th1: T-helper-1 cell

Th2: T-helper-2 cell

Th3: T-helper-3 cell

TLR-4: toll-like receptor-4

TNF: tumor necrosis factor

$T_{reg}1$: T-regulatory-like 1 cell

Chapter 1 – General introduction

I. Asthma

Atopic asthma is considered a type I hypersensitivity reaction that occurs in the respiratory tract and is associated with genetic and environmental determinants (1-3). The pathology involves the inhalation of an allergen that activates immunoglobulin E (IgE)-sensitized mast cells residing in the nasal or bronchial mucosa. This activation causes the release of pro-inflammatory mediators that activate other resident cells causing further mediator release (4, 5). Together these mediators cause airway inflammation, airway hyperresponsiveness (AHR), and mucus overproduction in the airways. A patient with asthma can have tightness in the chest and wheezing, and in more severe cases, gasp for breath (1-3). The worst attacks of acute asthma result in blockage of the airways that can be fatal (6). The airways of asthmatics are subject to airway remodeling (7-10). The airway epithelium can be stripped off by inflammation and the submucosa is thickened due to the deposition of collagen beneath the basement membrane. The smooth muscles surrounding the airways can be enlarged and are hyperresponsive to agonists leading to further narrowing of the airways.

The airways of asthmatic patients can be chronically inflamed with infiltration of numerous cell types, including: mast cells, basophils, eosinophils, lymphocytes, neutrophils, as well as NKT cells (1, 11, 12). Eosinophils are more than not found in the smooth muscle bundles of asthmatic bronchi along with elevated numbers of mast cells (13, 14). Over 50 separate mediators, cytokine and chemokine, have been associated with asthma and are found in the lung secretions of patients (1, 11, 12, 15-17).

Given this brief overview of the mechanisms involved asthma, what are some of the exact mediators that lead to the asthmatic phenotype and how do the aforementioned cell types interact to produce their release. Like all type I hypersensitivity diseases, asthma develops in two phases. The first is a sensitization phase and the second is an effector phase (1, 2, 18).

II. Hypersensitivity

In 1901, the first Nobel Prize for Physiology and Medicine was awarded to Emil von Behring for his work on serum therapy against diphtheria (19-21). He found that animals that had been immunized to diphtheria toxin became ill, or in the worse cases died, following a second dose of the toxin. This occurred even if the secondary doses were reduced to the point that they no longer had any effects on non-immunized animals. Behring named this heightened susceptibility to the diphtheria toxin "hypersensitivity" and reasoned that it was a result of a direct effect of the toxin. A similar effect was seen by Richet and Hericourt during an animal's secondary exposure to eel toxin and they surmised that the toxin was acting in a more potent manner in animals that had been previously primed to the toxin (22).

In the early 1900s people suspected that the cause of the hypersensitivity reactions was due to immune responses directed towards the toxin. The first evidence of this was shown by Richet and Portier in 1902 from their experiments to determine the strength of toxins from marine invertebrates in dogs (22, 23). They found that after the dogs were primed with the toxins, the smallest dose of the toxin, when administered again after several weeks, caused an immediate clinical shock syndrome that lead to the animal's death. More importantly they found that the hypersensitivity reaction could be transferred to naïve animals by the serum from the primed animals. This immune outcome was later termed allergy and those antigens that prime an individual were termed allergens.

It was not until 1967 that some of the precise molecular mechanisms surrounding hypersensitivity reactions were known (24). Researchers showed that the exact molecule in the serum that transferred sensitivity to an allergen was IgE (24). It was later found that the role of IgE was to bind to mast cells and trigger their activation in the presence of their specific allergen. The mediators released during activation caused allergic symptoms. Later studies showed that 4 categories of hypersensitivities existed.

The four types of hypersensitivity are: type I (IgE mediated hypersensitivity), type II (direct Ab-mediated hypersensitivity), type III (immune complex-mediated hypersensitivity) and type IV (delayed type cell-mediated hypersensitivity). All four hypersensitivities develop in two distinct phases. The first is referred to as the sensitization phase, while the other is called the effector/elicitation phase (18). The sensitization phase occurs during the primary immune response while the effector/elicitation phase is the secondary immune response.

Of these four types of hypersensitivity, I will focus on type I as this is involved in sensitization and effector phases of asthma.

III. Type I hypersensitivity

Allergic diseases such as asthma, eczema, hayfever, and anaphylaxis are all classified as type I hypersensitivities. Type I hypersensitivity is generally known as immediate type hypersensitivity because the secondary response to an allergen occurs rapidly, often within 30 min post encounter. These allergens are often soluble proteins that are associated with larger particles. Symptoms of type I reactions can range from itching and swelling (eczema) to difficulties in breathing (asthma) (1; 2, 12). In extreme of cases, shock and death can result (anaphylaxis). It is not currently known why a specific subgroup of individuals produce allergen-specific IgE that are characteristic of type I hypersensitivity reactions. Genetic factors are involved that make individuals susceptible (25-31). In addition the intrinsic characteristics of allergens must be important for allergic sensitization, since the number of allergens is limited compared to the total number of inhaled proteins (32-34).

i. Atopy and Allergic Diseases

Atopy was originally a term that was used to describe combinations of asthma, eczema, and hay fever that run in families. Today, any sensitization to an allergen that results in allergen-specific IgE production, tested by a positive skin test for anti-allergen IgE, is considered to be atopy (1, 2).

In individuals with atopy two types of secondary/effector reactions can take place, systemic and local. In a systemic atopic response (anaphylaxis) the entire body is affected that can lead to a sudden large drop in blood pressure and in extreme cases, death (35-37). In local atopy the reaction depends on the anatomical localization of the effect. For example, atopic asthma is inflammation in the airways that is a result of IgE-mediated responses to specific inhaled allergens (1, 2). Atopic rhinitis on the other hand is IgE inflammatory responses that occur in the nose (38-40). It should however, be stated that these diseases may be heterogeneous in their etiology. For instance asthma, in some cases, is not triggered by IgE-mediated mechanisms. Such patients are considered to have intrinsic asthma and have normal levels of IgE in their serum (41-43).

Atopy appears to be a disease that is predominately found in developed countries. In North America around 20% of the population is thought to be atopic. In Britain and Australia estimates have 20% of children under the age of 14 having atopic dermatitis while 25% are asthmatic (44). In Sweden the prevalence of asthma, allergic rhinitis, and eczema doubled from the 1980s to the 1990s (45). Furthermore, in developed nations, most of the morbidity and mortality associated with allergy is directly attributed to allergic asthma. Currently there is no cure for asthma, thus billions of dollars are spent treating the symptoms in children and adults (1, 2, 46-48). Moreover, the loss of workforce productivity related to atopy is considerable.

It has estimated that about 20 million Americans, around 7% of the population, currently have asthma. Furthermore, rates of asthma in females are 35% greater than in males (46). These numbers have been steadily increasing as it is estimated that the prevalence of the disease has increased by 75% from 1980-1994 (44). The increase in the prevalence of asthma caused 13.6 million visits to physician offices in 2004 in the United States (46-48). In 2001 approximately 2 million patients were admitted to emergency rooms, one-quarter of all emergency visits, because of asthma. This has a large economic impact as it is thought that the direct health care costs for asthma in the United States are more than $10 billion (48).

ii. Determinants of type I hypersensitivity

Genetics

The exact reasons why type I hypersensitivity reactions occur in only some individuals is unknown. However, genetics play a role (25-31). Families in which both parents are atopic have a 50% chance of offspring who are atopic as well (25, 26). This is compared to 30% in those families that only have one atopic parent and 19% in families in which both parents are non-atopic.

Family, twin studies, and cloning techniques have uncovered around 13 chromosomal regions and more than 20 genes that may contribute to the development and expression of allergic/atopic diseases (25-31). Of these the human chromosomal region 5q31-33 is of great interest as in contains a gene cluster that encodes many T-helper 2 (Th2) cytokines. These Th2 cytokines that promote the production of IgE and are integral in the development of atopy include IL-4 and IL-13. Interestingly patients with specific polymorphisms in this chromosomal region have greater numbers of Th2 cells residing in their tissues (25, 49, 50), that produce large amounts of IL-4 and IL-13 (49, 50). One particular polymorphism in the α unit of the IL-4 receptor (IL-4Rα) has been correlated with elevated levels of total serum IgE and the development of severe asthma (51, 52). Other genes that are located in this region that have also been correlated with the development of atopy and include GM-CSF (53, 54), CD14 (55, 56), TGFβ (57, 58), and glucocorticoid receptor (59, 60).

Along with the 5q31-33 locus, the major histocompatibility complex (MHC) region on chromosome 6 has consistently shown linkage to many atopic and asthma associated phenotypes (61-63). It is the MHC II complexes that Th2 cells interact with to produce their cytokines. The *HLA-DRB1* and *HLA-DBQ1* MHC II genes are strongly linked to IgE titres specific for many potent allergens as house dust mite (HDM) and common cat allergens (25, 61).

Determining the genes of importance in atopy is complicated as their expression is subject to specific modifications of the chromatin components. In these cases a specific genetic determinant is more likely to be expressed if inherited from the mother, while the allele from the father remains silent. Therefore an individual is more likely to harbor a specific atopic determinant if it was given to he or she by the mother rather than the father (25). As a result, an individual's "atopic genotype" will not necessarily correlate with the expressed "atopic phenotype". Another major difficultly in addressing genetic atopic determinants is that some alleles are only relevant in specific ethnic groups. An example is an allele for the high affinity IgE receptor (FcϵRIβ) that has been linked to the development of asthma in white South Africans, but was not associated with asthma in black South Africans (64). Black South African asthma patients had an entirely different association for the FcϵRIβ gene. Thus, although useful in making predictions in the development of atopic diseases, much remains to be elucidated in the genetics of atopy and asthma.

Environmental determinants

Although genetics play an important role in determining whether or not an individual will become atopic and develop type I hypersensitivity, it is not the sole determinant. Environmental exposure to specific protein antigens must also play a role as twin studies have shown that twin siblings only share responsiveness to the same allergens 60% of the time. Indeed, it has been noted that numerous conditions can be associated with the development and onset of type I atopic reactions. These include chronic illness, acute viral, bacterial, or fungal infections, emotional stress, fluctuating hormone levels, nutritional deficiency, as well as the presence of various pollutants (65-68). All of these factors may weaken the immune system thereby leaving the body more open to allergen entry and sensitization. However, it is likely that a combination of environmental and genetic factors predispose an individual to atopy.

IV. Phases in the Development of Type I hypersensitivity

IV. Phase 1: Sensitization Phase

The sensitization phase of a type I hypersensitivity reaction begins when a protein antigen penetrates the skin or a mucosal barrier. Following this initial penetration a number of cell types orchestrate sensitization. These include dendritic cells (DCs), $CD4^+$ T cells, and B Cells.

i. Cells critical in the Sensitization Phase

Dendritic cells (DCs)

DCs have long finger-like membrane processes that resemble the dendrites on neurons. DCs are derived from myeloid or lymphoid precursors in either the thymus or the bone marrow. Generally DCs that are newly produced in the bone marrow migrate through the blood and take up residence in peripheral tissue near mucosal sites where they remain in an immature state and wait to encounter protein antigens by endocytosis (69, 70).

One of the initial immunological events, following antigen penetration through the mucosal barrier is uptake by immature DCs. Once activated these DCs then migrate to the local draining lymph nodes (LNs) and present immunogenic peptides loaded on MHC II molecules to naïve CD4$^+$ T cells (71-75). The DCs then direct the T cells to differentiate into a specific Th subset. This subset is determined by the specific activating signals the DCs received in the tissue during their capture of the antigen. Therefore, DCs are crucial for determining the resulting immune response. In conjunction with their interactions with T cells when in the LNs, DCs also give captured antigens to B cells for the first stage of B cell activation (71-75). Although they share many features, multiple subtypes of DCs with distinct life span and immune functions have been identified (76, 77). The DC subtypes in mouse and in human include: type-1 interferon-producing plasmacytoid DCs (pDCs) and myeloid DCs (mDCs). Both subtypes of DCs are found in tissues, the circulation, and LNs.

CD4$^+$ T cells

Naïve CD4$^+$ T cells are bone marrow derived cells that develop in the thymus and reside in LNs. In LNs naïve CD4$^+$ T cells interact with the MHC II complexes of APCs, in most cases DCs, through their T cell receptor (TCR), differentiate and mount an immune response (78-80). This immune response is directed by the APC/DCs and can take the form of a Th1, Th2, or, in the case of mucosal immune tolerance, a regulatory T cell (Treg) response. These different Th responses are classified by the types of cytokines that the Th cells produce. In the case of type I hypersensitivity the immune response is of a Th2 phenotype (78-80).

Th2 cells are able to produce and secrete cytokines such as IL-13, IL-4, IL-5, IL-6, IL-10, and IL-13 (78-80). Along with the CD40-CD40L and icos-icosL co-stimulatory pathways these Th2 cells secrete IL-4, IL-13, and IL-5 (81, 82). IL-4, IL-13 and IL-5 induce isotype switching in the B cells to IgA, IgE, and IgG_1 in mice and in humans IgG_1 is replaced by IgG_4 (78-80).

B cells

B cells are bone marrow derived lymphocytes whose development is categorized into two stages. The first of these phases is the maturation phase where the hematopoietic stem cell matures into naïve B cells (83-87). In the second phase allergen-activated B cells differentiates into Ab secreting plasma cells and memory B cells; currently we suspect that three signals are involved in such B cell development (88-96).

The three signal model of B cell activation begins with the naïve B cell interacting with specific protein antigen through the B cell receptor (BCR). The BCR on naïve B cells is composed of a IgM (mIgM) that is non-covalently complexed with the Igα and Igβ accessory signaling proteins. This naïve B cell is believed to be in a cognitive state as their BCR is able to recognize and react to specific protein antigen. This antigen is usually provided in the LNs by activated DCs derived from peripheral sites. Once a specific protein antigen is bound to the BCR a signal is sent through Igα and Igβ signaling molecules. Following binding to BCR, the antigen-BCR complex is internalized into the B cell and processed into antigenic peptides and loaded on

MHC II complexes. These antigenic peptides are then recognized by TCRs on activated T cells in the LN. During this engagement, CD40L on the surface of activated T cells stimulates CD40 on B cells and this CD40-CD40L interaction constitutes the second signal. The third signal comes from specific cytokines released by T cells during their engagement with B cells. Following this final activation step B cells are fully activated, expand and upon re-stimulation by activated T cells differentiate into Ab secreting plasma cells. As mentioned previously for type I hypersensitivity reactions one of the most important Ab secreted is of the IgE isotype and is a result of the Th2 cell cytokines IL-4, IL-13, and IL-5.

ii. Important mediators in the Sensitization Phase

IL-4

IL-4 is primarily secreted by CD-40L cross-linked Th2 $CD4^+$ T cells (97-99). However; it can also be produced by basophils (100, 101), mast cells (97, 100, 102), and NKT cells (103, 104). The actions of IL-4 are in most cases antagonistic to those of the generally thought of Th1 cytokine, IFN-γ. Because its receptor is widely expressed in the body, IL-4 affects many cells and tissues (105, 106).

IL-4 is of critical importance for the differentiation and growth of Th2 cells. It is critical for the development of an IgE response directed to pathogens such as helminths (107, 108). IL-4 stimulates differentiation and growth of B cells, and causes up-regulation of MHC II (109). It also promotes isotype switching in mice to IgA, IgG_1, and IgE, while inhibiting switching to the IgG_{2a}, IgG_{2b}, and IgG_3 isotypes. IL-4 is a growth factor for mast cells, an important cell type in the effector stage of type I responses through their binding of IgE (97-99). Thus, IL-4 plays significant roles in allergic responses.

IL-4, as mentioned above, is important for host defenses towards helminths. IL-4 secreting Th2 cells induce eosinophils to express the low affinity IgE receptor FcεRIIB (CD23). This allows eosinophils express antibody-dependent cell-mediated cytotoxicity (109, 110). In macrophages, IL-4 is able to inhibit the secretion of pro-inflammatory chemokines and cytokines such as TNF and IL-1β, as well as impeding the production of reactive oxygen and nitrogen intermediates (109, 111).

IL-13

IL-13 is secreted by CD4$^+$ Th2 cells but also can be secreted by basophils (112, 113) and NKT cells (112, 114). Its biological functions are closely related to IL-4 (112). Activation of B cells in the presence of IL-13 is able to help stimulate proliferation and induce an increase in surface expression of CD23, MHC II, and IgM (112, 115, 116). IL-13 plays a role in B cell isotype switching to the IgE and IgG$_1$ subtypes (117). For example, IL-4 deficient mice are still able to produce IgE. This means that there is an IL-13-dependant pathway separate from that of the IL-4-dependent pathway (112, 118, 119). Furthermore, CD4$^+$ Th2 cells from IL-13 deficient mice produce significantly lower amounts of the Th2 cytokines IL-4, IL-5, and IL-10. As a result these mice have lower serum levels of IgE (120). IL-13 also has an important role in AHR as it causes AHR when administered into the airways of mice (112, 121). It is also able to heighten the bronchial smooth muscle's responses to histamine and leukotrienes (112).

iii. Cellular mechanisms in the Sensitization Phase (Fig. 1.1)

Type I hypersensitivity reactions are initiated when an antigen crosses the mucosal boundary and is endocytosed by immature DCs (iDCs) (Fig. 1.1 #1). Before or while being endocytosed, these antigens cause currently unknown "danger signals" that activate DCs. Once activated DCs cleave the antigens into immunogenic peptides during their migration back to the local draining LNs (Fig. 1.1 #2). Once in the LNs DCs present the processed protein antigen on their MHC II complexes to naïve CD4$^+$ Th cells (Th0) causing them to differentiate into CD4$^+$ Th2 cells (Fig. 1.1 #3a). For this differentiation IL-4 and IL-6 are required. They also pass some of the antigen to naïve B cells (nB) which leads to the first of three stages in B cell activation and differentiation (Fig. 1.1 #3b). This causes B cells to endocytose and process the antigen in the same manner as the DCs and present the immunogenic peptides on their MHC II complexes. Newly activated CD4$^+$ Th2 cells then interact with immunogenic peptides on B cells' MHC II, providing the second and third signals to the B cells via co-stimulation (CD40-CD40L and iCos pathways) and the release of cytokines (IL-4, IL-13, and IL-5) respectively (Fig. 1.1 #4). Once the three signals are received, the B cell is fully activated (AB). If this all unfolds the protein antigen is defined as an allergen (12, 18).

These newly activated allergen-specific Th2 and activated B cells can up-regulate the expression of tissue-specific homing receptors that cause Th2 and B cells to migrate to the tissue in which the allergen first produced the "danger signal" (Fig 1.1 #5). In the tissue Th2 and B cells coordinate a primary immune response. During this primary response the allergen-specific B cells recognize the allergen through their BCR and uptake and process it into immunogenic peptides and present it on their MHC II complexes. The peptides loaded on the MHC II are recognized by allergen-specific Th2 cells and, through the release of IL-4, IL-13, and IL-5, influence the B cells to differentiate into Ab secreting plasma cells (Fig. 1.1 #6). These plasma cells (PC) secrete allergen-specific IgE along with some IgA and IgG_1. The IgE produced by these plasma cells enters the lymphatics and circulates in the blood (Fig. 1.1 #7). This IgE encounters basophils (Bas) in the blood (Fig. 1.1 #8) and mast cells (MC) in the tissue (Fig. 1.1 #9) and binds to the high affinity IgE receptor FcεRI that is highly expressed on the surface of these cells. The basophils and mast cells are sensitized (SBas and SMC) which readies them for the early phase reaction of the effector phase (12, 18).

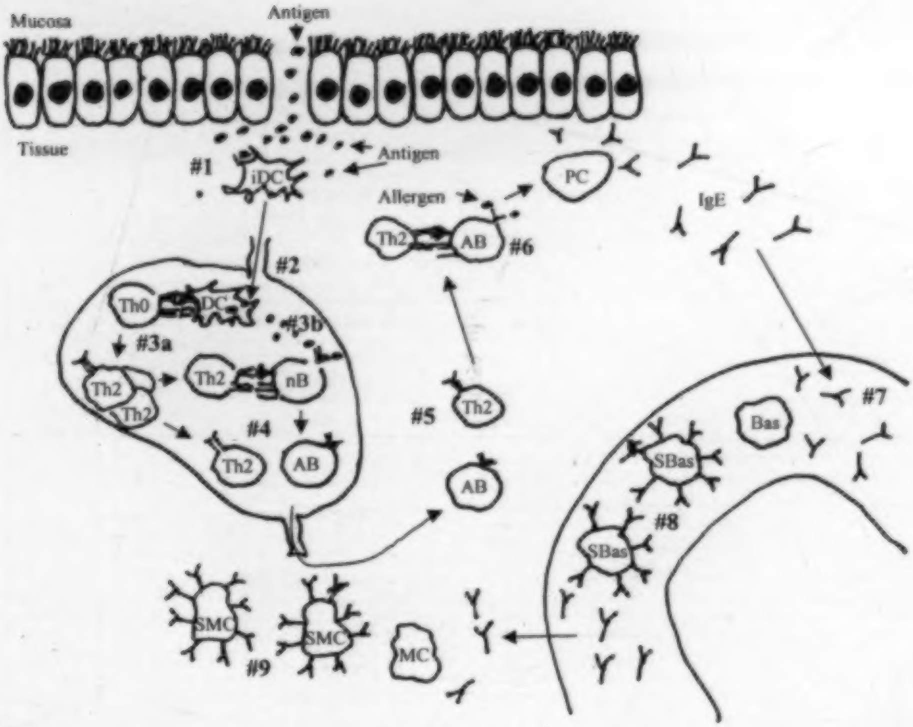

Figure 1.1. Cellular mechanisms of the Sensitization Phase of Type I Hypersensitivity. iDC = immature DC, Th2 = T-helper-2 cell, nB = naïve B cell, AB = activated B cell, PC = plasma cell, Bas = basophil, SBas = sensitized basophil, MC = mast cell, SMC = sensitized mast cell

To become an allergen the protein antigen, or substances inhaled with the antigen, must be able to produce the initial "danger signal" that directs an atopic/allergic immune response. Why particular antigens are favored for allergic sensitization is unknown, but intrinsic characteristics must be important.

iv. Danger signals that lead to atopic sensitization

The so called "danger signals" may be elicited by mediators released as a result of the activation of some sort of receptor system(s) in the airway mucosa. This activation could be by allergens themselves or by substances that are inhaled or encountered along with the allergens.

Lipopolysaccaride (LPS) and TLR-4 interaction

LPS, a cell wall component of Gram-negative bacteria, is widespread in the environment including household dust and in the air we breathe. Polymorphisms in the CD14 gene, part of the TLR-4 complex that recognizes LPS, have been shown to be linked to the development of asthma. Furthermore, infections in the respiratory tract have been linked to the severity of atopy and asthma (122-125). Some have speculated that the opposing roles of LPS in allergic diseases could be explained by differences in the patient's exposure levels to the LPS (126). Eisenbarth et al showed that allergic sensitization towards inhaled OVA occurred if it was given with LPS and this Th2 response required signaling through the TLR4 complex (127). Furthermore, these authors showed that differing doses of LPS induced different immune responses. Spenocytes from mice given low dose of LPS with OVA had high IL-5, IL-4 and IL-13 production, while spenocytes from mice given high dose of LPS with OVA had high IFN-γ production indicating a Th1 immune response. It is unclear why a lower dose of LPS favors Th2 induction, but it could be due to the exact cytokine milieu in the airway mucosa following LPS exposure. Interestingly, LPS can induce release of TNF from macrophages (128). Macrophages are abundant in the airways and TNF has strong immunogenic properties.

TNF could be the possible "danger signal" in the sensitization phase that starts the immune response. Furthermore, TNF can induce the release of GM-CSF by various cell types including airway epithelial cells and has been show to promote allergic sensitization when expressed in the airway. This shows that along with direct effects TNF could be initiating immune responses through indirect mechanisms.

GM-CSF expressed in the airways

In addition to its effects on hematopoiesis of granulocyte and monocyte lineages, GM-CSF can exhibit adjuvant-like properties *in vitro* and in *in vivo* (130-136). In 1987 Morrissey et al showed the *in vitro*, GM-CSF enhanced primary immune responses towards sheep red blood cells (130). Moreover, in some animal models, GM-CSF increased immunogenicity of tumors (134-136) Moreover, clinical trials have assessed GM-CSF as an adjuvant for tumor vaccinations (137, 138). Furthermore, GM-CSF can activate bone marrow derived macrophages to upregulate expression of MHC class II molecules. The immunosuppressive activity of alveolar macrophages has also been shown to be inhibited in an *in vitro* setting by GM-CSF (129). This in turn greatly increases their capacity to act to present antigen to T cells (131). Along with macrophages, GM-CSF can up-regulate expression of costimulatory molecules on DCs (133). Interestingly GM-CSF promotes allergic sensitization in a mouse model of asthma.

GM-CSF can change the pulmonary micro-environment in a way that promotes allergic sensitization to OVA. When the the authors induced transgene expression of GM-CSF in the airways it acted as a signal to promote allergic sensitization to OVA given into the airways (139). The result was eosinophilic inflammation in perivascular and peribronchial regions of the lung. The responses were MHC II dependent, as the GM-CSF stimulated the expansion of macrophage and DC populations. This, the authors argued, induced OVA-specific, T cell-mediated immunity that favored a Th2/allergic phenotype. The above studies have identified possible mechanisms whereby potent allergens could promote allergic sensitization and development of asthma.

v. Allergen biology

Common characteristics of potent allergens

When looking for characteristics that many potent allergens share we find that they posses intrinsic serine proteinase activity themselves or are inhaled in particles that are rich in serine proteinases. This proteinase activity is integral to their immunogenic properties. Potent allergens from house dust mite and fungi that possess proteinase activity are significant modulators of airway epithelial function (140-143). The proteinases originating from house dust mites (HDM) *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* include group 1 cysteine proteases (144), group 3 trypsins (145), group 6 chymotrypsins (146), and the recently identified group 9 collagenolytic serine proteases (147). These proteinases, either working alone or synergistically increase the permeability of epithelial cell layers *in vitro* by their ability to degrade the integral tight junction proteins ZO-1 and desmoplakin (148, 149). These proteinases also stimulate airway epithelial cells directly to produce the inflammatory cytokines IL-6 and IL-8 (143). These proteolytic activities could have a couple of important functions. They may facilitate access of the inhaled allergen to DCs situated below the airway epithelial layer. The second is that through the release of specific mediators they may modulate the functions of immune cells. Of the potent allergens the best studied to date is the proteinase activity of the HDM allergen, *Der p 1*. The proteinase activity of *Der p 1* augments the permeability of the bronchial epithelium. This may increase antigen uptake and processing by DCs beneath the epithelial layer (144). Furthermore, its proteinase activity can elicit release of proinflammatory cytokines that could act as "danger signals" (144). The proteinase activity of *Der p 1* is also able to cleave CD23, the low-affinity IgE receptor (FcεRII), as well as CD25, the IL-2 receptor α-chain, expressed on the surface of activated T cells and highly expressed on the surface of T_{reg} cells (150, 151). Such cleavage could possibly lead to dysregulation of the immune response and enhanced production of IgE (152). Other proteinases may use at least some of these strategies to similar effect. Interestingly, a proteinase-mediated pathway has been implicated in the development of Th2 allergic disease.

Proteinase-mediated pathway in the development of allergic disease

Kheradmand et al have identified that a variety of common potent allergens have proteinase activity that underlies their ability to promote allergic sensitization (153). They identified three potent fungal allergens that contain active proteinases, as well as amylase, activities. Allergens were prepared from *A. fumigatus*, *A. oryzae* and the pollen of *Ambrosia artemisiifolia* (ragweed). These allergens were tested for their ability to induce allergic sensitization and development of asthma when administered into the airways of mice. The allergens were administered over a range of proteinase activities and promoted allergic sensitization to OVA administered into the airways, along with fungal allergens and a pure fungal proteinase extract from *A. fumigatus*. OVA administered alone did not cause allergic disease in the airways. In all cases it was only the active proteinase that was a necessity for the development of allergic sensitization and subsequent development of allergic disease. From these results the authors concluded it was the proteinase function of these fungal allergens that were essential to promote allergic sensitization.

The proteinase activity of the allergens could mediate the "danger signal" through the activation of receptors that sense proteolytic activity. One such family, the Proteinase-Activated Receptors (PARs), has four members (PAR-1 to 4) and is able to recognize proteinase activity. One member of this family PAR-2 is of particular interest as it has been shown to be activated by many common allergens. Furthermore, its activation promotes release of inflammatory mediators. These mediators could initiate immunogenic responses towards the allergen inhaled in particles rich in proteinase activity.

V. Proteinase-Activated Receptors (PARs)

i. PAR activation

Serine proteinases comprise a large family of enzymes that are characterized by a reactive serine residue side chain in the active site. Like many other proteinases they serve important and diverse biological functions. One of these functions is to act as signaling molecules that regulate cellular processes. This is accomplished by their activation of seven-transmembrane G-coupled PARs. A wide range of serine proteinases are able to cleave and thus activate PARs. These include proteinases from the coagulation cascade, as well as those from inflammatory cells. PAR-2 can be activated by many potent allergens that are proteinases themselves. The mechanism by which the proteinases cleave and activate the PARs is similar. First the proteinase cleaves at a specific cleave sites within the extracellular N-terminus of the receptor that exposes a new amino terminus that serves as a tethered ligand domain. This tethered ligand domain then folds back on the receptor and binds to conserved regions on the second extracellular loop of the receptor. The binding of the tethered ligand to the second extracellular loop activates the PAR (Fig. 1.2A). One can also activate PARs by using synthetic activating peptides (PAR-AP) that have sequence identity with the tethered ligand sequences. These activating peptides bind directly to the second extracellular loop of the receptor, thus activating it (Fig. 1.2B).

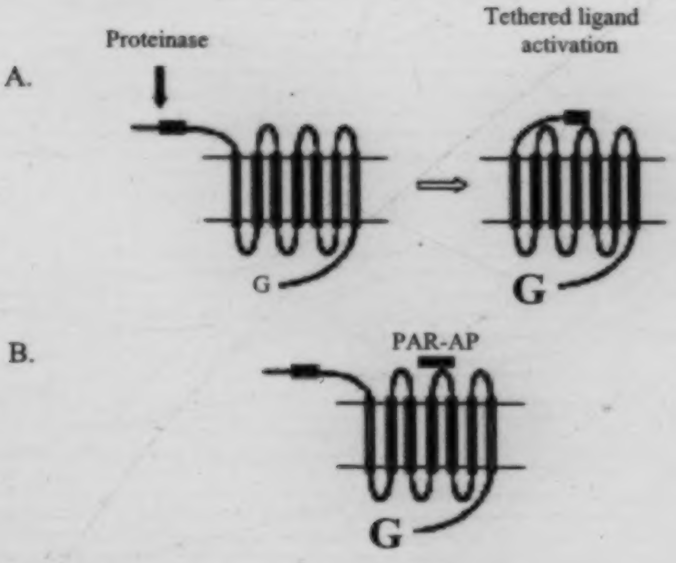

Figure 1.2. Mechanisms of PAR activation.

Large G denotes an activated PAR

As mentioned the PAR family of receptors has four members PAR-1 (also known as the thrombin receptor), 2, 3, and 4. I will focus on PAR-2, as there is evidence that the activation of PAR-2 is important in the sensitization phase of type I reactions like asthma.

ii. PAR-2

Expression of PAR-2 in the body

PAR-2 is expressed in the brain, eye, airways, heart, GI tract, pancreas, kidney, liver, prostate, ovary, testes, and skin (154-157) (158-161). It is also expressed in epithelial cells, macrophages, endothelial cells, smooth muscle cells, osteoblasts, and immune cells such as T cells, neutrophils, mast cells, and eosinophils (162-171). Of all of the cell types that express PAR-2, it is likely that inhaled proteolytic allergens interact with macrophages in the airways and also airway with epithelial cells.

Allergens – exogenous proteinases in allergic type I sensitization

Many of the potent allergens that we become sensitized have proteinase activity. These include allergens from house dust mites (HDM), cockroaches, molds, pollen, and fungi. Such proteinase activity could enable these allergens to penetrate the mucosa and make them more accessible to DCs situated under the epithelial layer. These proteinases could release specific mediators that modulate DC function in a way that could promote atopic sensitization. Such activities are seen with one of the common allergens from HDM. Along with aiding in the penetration of the mucosa, the proteinase activity of these allergens could mediate some effects through activation of the PAR-2 receptor. *Der p 1* is able to cause the release of inflammatory mediators that could act as the initial "danger signal" that would begin an immune response towards the allergen. This "danger signal" could be elicited as a result of PAR-2 activation by *Der p 1*.

In addition to *Der p 1*, other allergens from mites can mediate their effects through the PAR-2 receptor. These include *Der p 3, 6, and 9,* although there does not appear to be a consensus on all of these allergens. Along with *Der p* extracts from cockroachand fungi, which have proteinase activity, can activate PAR-2. Although there are many potent allergens that have intrinsic proteinase activity, or are inhaled in particles rich in proteinases, only a select few have been tested as to whether or not they active PAR-2. A broad-spectrum study involving many of these proteolytic allergens and their ability to activate PAR-2 would be interesting.

Activation of PAR-2 could be important in initial sensitization through the release of specific mediators. Interestingly, along with its potential as a recognition receptor for potent allergens and having an important role in allergic sensitization, PAR-2 appears to be important in the effector phase of type I reactions. This is through its activation by endogenously released proteinases during allergen challenge.

VI. Phase 2 of Type I hypersensitivity: Effector Phase

When an allergen enters the body for a second or subsequent time and encounters mast cells and basophils loaded with allergen-specific IgE the effector phase of the type I hypersensitivity reaction is triggered (12, 18). The allergen causes the mast cells and basophils to be activated, resulting in the immediate release of mediators into the surrounding tissue. This causes a rapid onset of symptoms and is termed the "early phase reaction" of the effector phase. These and other mediators then cause leukocyte inflammation in the tissue that in turn release more inflammatory mediators in the "late phase reaction".

VI. a. Early phase of the effector phase

a. i. Cells critical in the early phase reaction of the effector phase

Mast Cells

Mast cells are bone marrow derived hematopoietic cells that enter tissues by chemotactic factors such as IL-8, SCF, SDF, TGFβ, RANTES, and eotaxin (172-177). Once in the tissue they differentiate under the influence of many factors with the most important being SCF. Mast cells are abundant in skin and connective tissues surrounding nerves, blood vessels, glandular ducts, and in mucosal sites (172-177). In the lungs mast cells are found in the bronchial connective tissue and in the alveolar spaces (175). Mast cell activation historically was thought to combat parasitic infection (178, 179). Upon activation mast cells release a plethora of mediators that induce symptoms designed to expel the parasitic pathogens. These include histamine, serotonin, leukocyte chemotatic factors, proteinases, tumor necrosis factor (TNF), various interleukins, platelet activating factor (PAF), leukotrienes, and prostaglandins to name a few (178-181). Of these, the primary mediators of allergic symptoms in the early phase response are histamine and leukotrienes. Other important and key mediators are a number of proteinases such as mast cell tryptase (182-184). The fact that many of these mediators are preformed in the mast cell granules accounts for the immediate nature of type I hypersensitivity responses. In addition to mediators that are preformed in their granules, mast cells produce and secrete a large number of cytokines (IL-1, 2, 3, 4, 5, 6, 13, 16, TNF), chemokines (IL-8, MIP-1α, eotaxin, MCP-1), and growth factors (GM-CSF, PDGF) following degranulation (175, 178-181). These mediators play important roles in the late phase response of type I hypersensitivities.

a. ii. Important mediators in the early phase reaction

TNF

Originally known as TNFα, TNF was first discovered as a substance that was able to induce hemorrhagic necrosis of certain tumors in mice. Today TNF is widely known as a major inflammatory mediator induced by a number of mechanisms including by gram-negative bacteria. Depending on its concentration, TNF can have many effects in the body. Along with its role in inflammation, TNF possesses potent immunoregulatory and cytotoxic effects.

At lower concentrations TNF promotes up-regulation of adhesion molecules on endothelial cells, neutrophils, lymphocytes, and macrophages (185-187). This up-regulation of adhesion molecules leads to increased leukocyte extravasation and migration during inflammatory events. TNF also stimulates neotrophils, eosinophils, and macrophages to carry out their microbicidal activities (185, 186, 188).

At concentrations usually induced by infections that have not been contained, TNF circulates in the blood and acts like a hormone. This results in numerous cells throughout the body being affected. For instance TNF can act on cells in the brain to promote fever and also induces hepatocytes to produce acute phase proteins (189). In addition, TNF has proliferative effects on endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells (185-188, 190). TNF causes the production of collagen and fibrosis (191, 192). Such conditions are seen regularly in chronic inflammatory diseases such as asthma (1, 3, 11).

Effects of TNF can be lethal. For instance an infection of gram-negative bacteria can lead to large amounts of lipopolysaccaride (LPS) stimulating the immune system, which in turn leads to large quantities of TNF being released throughout the body (185). TNF at high levels exerts metabolic and circulatory effects that lead to endotoxic shock.

TNF: immune regulation

TNF resembles IL-1 in many immunoregulatory fashions. TNF can be expressed in the thymus and promote either survival or death of certain T cell lineages (193). TNF appears to be dispensable as TNF knock out (KO) mice have normal numbers of T cells (185, 193). Along with its ability to function within the thymus, TNF has potent immunogenic properties on DCs (194-196).

TNF: cytotoxic effects

TNF, along with its ability to induce necrosis in tumors, can kill many types of tumor cells by the induction of apoptosis (185). Indeed, a large component of activated macrophage and NK cell tumor cell killing is thought to be through their ability to secrete TNF. However, it is still unknown why TNF is unable to induce death in many cells that are normal and not of tumor lineage.

Histamine and Leukotrienes

Although they are different mediators histamine and leukotrienes have many similarities. Both are produced by mast cells and basophils and are released following their activation (181, 190, 196). Once released histamine and leukotrienes share many of the same physiological functions. Both are potent vasodilators, increase blood vessel permeability, induce contraction of bronchial smooth muscle (which leads to one of the cardinal feature of asthma, airway hyperresponsiveness (AHR)), increase mucus production in the airways (asthma), and stimulate sensory nerves (1, 11).

Proteinases

Interestingly, important mediators released during the early phase reaction of type I hypersensitivity are various proteinases. Many proteinases are released during mast cell activation and include mast cell tryptase, chymotrypsin-like proteinases, carboxypeptidase A3 (CPA3), granzyme B, cathepsin G, neuropsin, transmembrane tryptase, tryptase γ, proteinase serine member S (Prss) 31, MC proteinase (MCP) 1–10, and MCP-11/Prss34 (182-184). Four of the 15 serine proteases in mouse MCs are tryptases (designated MCP-6, MCP-7, Prss31, and Prss34). Their genes reside at chromosome 17A3.3 within a larger 11.8-megabase serine proteinase gene cluster that encodes 13 functional trypsin-like proteinases.

These proteinases have various functions including promoting vascular permeability, tissue and vascular remodeling, promoting the pathological features of asthma (airway inflammation and AHR), and important functions in innate immunity (1, 11, 182-184).

a. iii. Cellular mechanisms in the early phase reaction of Type I Hypersensitivity (Fig. 1.3)

The early phase reaction of the effector stage of type I hypersensitivity begins with the allergen contacting the same mucosal barrier it encountered during the sensitization stage (Fig. 1.3 #1). The allergen then causes the cross-linking of allergen-specific IgE bound the mast cells (11, 12, 18). This cross-linking induces mast cell activation and mediator release into the surrounding tissue (Fig. 1.3 #2). The primary mediators that are stored in the granules and induce allergic symptoms are histamine, TNF, and various proteinases. Leukotrienes, although not stored in the granules, are produced shortly after degranulation. The released histamine and leukotrienes bind to their receptors on smooth muscle cells that surround the blood vessels, causing them to relax (Fig. 1.3 #3). This increases blood flow to the affected area. In asthmatic patients both histamine and leukotrienes cause smooth muscles surrounding the bronchials to contract (Fig. 1.3 #4). TNF also begins to act on the endothelium resulting in up-regulation of ICAM-1, ICAM-2, VCAM-1, E and P-Selectin adhesion molecules (Fig. 1.3 #5). This up-regulation of adhesion molecules allows for the beginning of the selective recruitment of eosinophils, neutrophils, sensitized basophils, and $CD4^+$ Th2 cells into the inflamed tissue (Fig. 1.3 #6). TNF also causes release of other mediators such as GM-CSF that acts as a chemo-attractant for monocytes such as natural killer T cells (NKT) (Fig. 1.3 #7). Finally mast cell proteinases are able to mediate many of their effects through the PARs that are found on the surface of numerous cells including eosinophils, endothelium, smooth muscle, epithelial cells and neurons. PAR activation is able to cause numerous pathogenic mechanisms ranging from potentiating histamine-induced bronchial smooth muscle contractions, as well as cytokine expression that enhances basophil and eosinophil inflammation further, to stimulate release of neuropeptides from PAR-2 activated neurons involved in vasodilatation (Fig. 1.3 #8). With eosinophils (Eo), neutrophils (Ne), sensitized basophils (SBas), $CD4^+$ Th2 cells and NKT cells brought into the area of allergen recognition by mast cells the late phase reaction takes place.

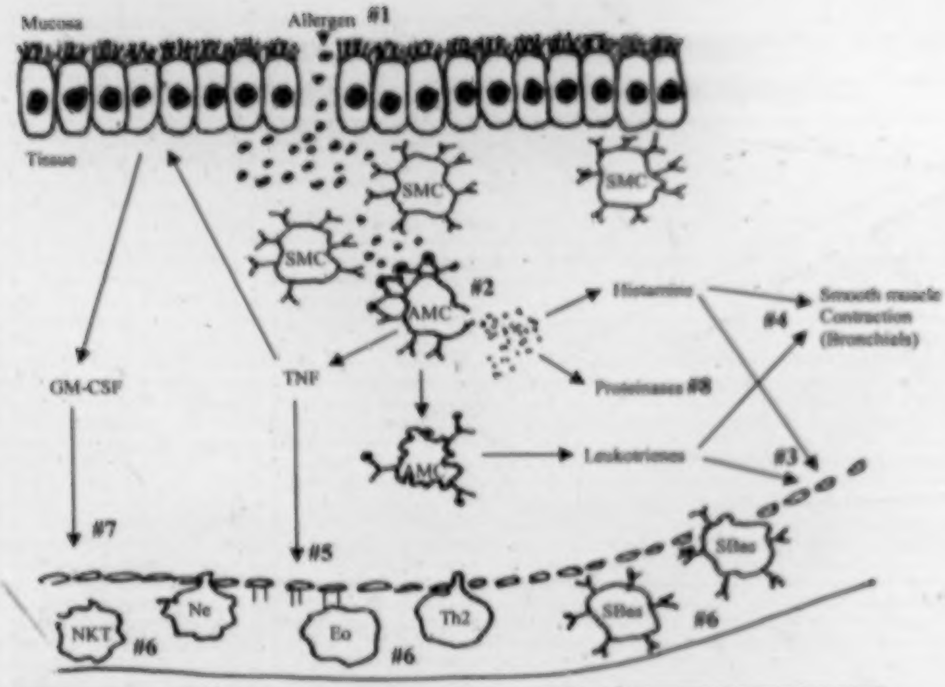

Figure 1.3. Early phase reaction of the effector phase of Type I Hypersensitivity.

SMC = sensitized mast cell, AMC = activated mast cell, NKT = natural killer T cell, SBas = sensitized basophil, Th2 = T-helper-2 cell, Eo = eosinophil, Ne = neutrophil.

VI. b. Late phase reaction of the effector phase

b. i. Cells critical in the late phase reaction of the effector phase

Basophils

Basophils are bone marrow derived hematopoietic cells that, although they are alike to mast cells have not been as intensely studied as them. In contrast to mast cells that reside near mucosal epithelial surfaces, peripheral nerves, blood vessels, etc, basophils are for the most part found in the blood and must be recruited to tissues by the inflammatory process (172, 197, 198). It is thought that the immediate response induced by mast cell activation causes sensitized basophils to migrate into inflammed sites (197, 198). Like mast cells basophils bind IgE through their high affinity FcεRI receptors and when an allergen binds to the allergen-specific IgE it aggregates the high affinity receptors resulting in degranulation. Degranulating basophils release many of the mediators that mast cells do, but they have been shown to release larger amounts of IL-4 and IL-13 (1, 2, 197, 198). Interestingly in atopic individuals basophil granules also have large quantities of proteinase such as tryptase (184, 199).

Eosinophils

Eosinophils are leukocytes with bi-lobed nuclei and large cytoplasmic granules that contain highly basic proteins and enzymes that are effective in the killing and clearance of large parasitic organisms (200, 201). Along with the ability to clear parasites, eosinophils also have limited phagocytic function. The majority of mature eosinophils reside in connective tissue and thus constitute a small proportion of all leukocytes in the blood. Activated eosinophils have been shown to express FcεR, FcαR, and FcγR that are able to bind antigen-specific IgE, IgA, and IgG respectively (200-202). When activated, eosinophils are able to degranulate and release many inflammatory mediators. These include PAF, leukotrienes, major basic protein (MBP), IL-4, IL-10, eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (200, 202-204). MBP has potent activity against parasites such as helminthes, but in the case of asthma it is toxic to respiratory cells, causing desquamation, and can induce asthma-like symtoms in animals when administered into the airways. ECP and EDN both have strong ribonuclease activity that is effective against viruses, but in asthmatic airways, like MBP, are damaging to the airway epithelium.

Neutrophils

After growing to maturity in the bone marrow neutrophils enter the blood where they have a life span of 1-2 days. The major role of neutrophils is that of pathogen clearance by phagocytosis (205). When they reach the site of pathogen infiltration they begin to phagocytose foreign cells or macromolecules (205). Neutrophil cytoplasmic granules then fuse with the phagosomes and the contents are released into the vesicle. The phagosomes are flooded with hydrogen ions that lower the pH of the vesicle that results in the hydrolysis of the foreign body. This low pH also activates many of the numerous proteinases that are contained within the granule that aid in clearance as well. Along with the granule enzymes oxidative molecules, such as neutrophil myeloperoxidase, play an important part in pathogen clearance. Following their activation by mediators such as IL-8, it is these oxidative molecules and lytic enzymes that contribute to tissue damage in type I reactions such as asthma (205-207).

NKT cells

NKT cells are of the lymphoid lineage that has functional characteristics of both Natural killer (NK) cells and T cells (208). NKT cells are distributed throughout the body in low numbers. Following activation NKT cells migrate to the sites of inflammation. *In vivo* NKT cells' most important effector function seems to be that of cytokine secretion. NKT cells carry with them preformed mRNAs for a collection of cytokines. These include IL-2, IL-4, IL-13, IL-10, and IFN-γ. NKT cells express on their surface a semi-invariant TCR that is able to recognize glycolipids presented on the MHC-like CD1d complex (208, 209). Once triggered via this semi-invariant TCR, NKT cells produce cytokines in differing quantities within 1-2 hours without having to differentiate into a Th-like effector cell.

Although not completely clear, NKT cells appear to play an important role in the late phase reaction of type I hypersensitivity reactions (209-211). This role appears to be that of producing large amounts of IL-4 and IL-13 (114, 210, 211). Indeed mice that are deficient of either NKT cells or the CD1d complex show reduced allergen-induced airway inflammation (114). This reduced airway inflammation can be reversed when NKT cells are reintroduced back into the NKT deficient mice.

b. ii. Important mediators in the late phase reaction

IL-3

IL-3 is produced for the most part by activated CD4$^+$ T cells and appears to be an essential growth and survival factor for mast cells, basophils, and eosinophils *in vivo* (212, 213). Furthermore, it also seems to be important in the responses of these cells during parasite infections. For instance IL-3 is able to regulate leukotriene pathways in mast cells and eosinophils in a way that potentiates leukotriene release. In the late phase response it is able to activate as well as promote the recruitment of mature, sensitized basophils.

GM-CSF

Systemically GM-CSF is able to act on bone marrow to increase the generation of hematopoietic precursor cells, in particular inflammatory leukocytes, from their immature precursors (212-215). GM-CSF is able to activate macrophages and is thought to play an important role in DC development and differentiation (214). More specifically it is thought that DCs differentiate from an immature to a mature state while in the presence of GM-CSF (216, 217). It has also been shown to promote the survival of eosinophils and have a role in their activation (214, 215, 217). This is of major importance in discussing it role in the late phase response where eosinophils are thought to play a vital role. As previously mentioned of the major mediators of inflammation, TNF is able to induce the production of GM-CSF in many cell types.

b. iii. Cellular mechanisms in the late phase reaction of Type I Hypersensitivity (Fig. 1.4)

Around 4-6 hours following the start of the effector stage of the type I hypersensitivity reaction the accumulation of inflammatory mediators released during the early phase causes a host of immune cells to infiltrate the tissue (11, 18). This infiltration began in the early phase and consists mostly of eosinophils and neutrophils. However, sensitized basophils, $CD4^+$ Th2 cells and NKT cells also make up this infiltrate. Along with the allergen, cytokines and chemokines produced and secreted by these cell types create an environment in which mast cells and basophils are activated both by IgE-dependent and IgE-independent mechanisms (11, 18) (Fig. 1.4 #1). Mast cell derived IL-5, IL-3, and GM-CSF are able to promote the activation of eosinophils and macrophages, while IL-8 is able to stimulate neutrophils (11, 18) (Fig. 1.4 #2). When activated the macrophages and neutrophils release proteolytic enzymes, and oxidative species that lead to further tissue damage in the inflamed site (11, 18) (Fig. 1.4 #3). Activated basophils undergoing degranulation release more leukotrienes, and large amounts of IL-4, IL-13. IL-4 enhances all Th2 responses in the tissue, while IL-13 heightens the bronchial smooth muscle responses to the leukotrienes as well as enhancing Th2 responses (11, 18). The proteinases released activate PARs in the tissue leading to enhancement of cytokine activity.

Activated eosinophils up-regulate their FcεR and FcαR receptors and bind allergen-specific IgE and IgA respectively. Upon cross-linking of these Ab the eosinophils undergo degranulation and release many inflammatory mediators (11, 18). These include leukotrienes (late phase AHR, mucus production, vasodilatation), MBP (airway epithelial cell damage/desquamation), EDN (damage to the airway epithelium), ECP (damage to the airway epithelium), IL-4 and low levels of IL-10 (further enhances Th2 environment) (11, 18) (Fig. 1.4 #4).

During this cellular activation lung DCs take up allergen and present it in MHC II complexes, and take up glycolipids released from tissue damage and present them in their MHC-like CD1d complexes. This results in the activation of allergen specific $CD4^+$ Th2 cells (Fig. 1.4 #5) and NKT cells (Fig. 1.4 #6) respectively. The activated $CD4^+$ Th2 cells produce IL-4 and IL-5 which help to activate eosinophils while the activated NKT cells produce large amounts of IL-4 and more importantly IL-13 that as mentioned previously is integral in mediating AHR (11, 18).

In chronic atopic asthmatic patients the sensitization and effector phases keep rotating leading to the hallmarks of asthma namely, AHR, mucous secretion, airway remodeling, and smooth muscle enlargement.

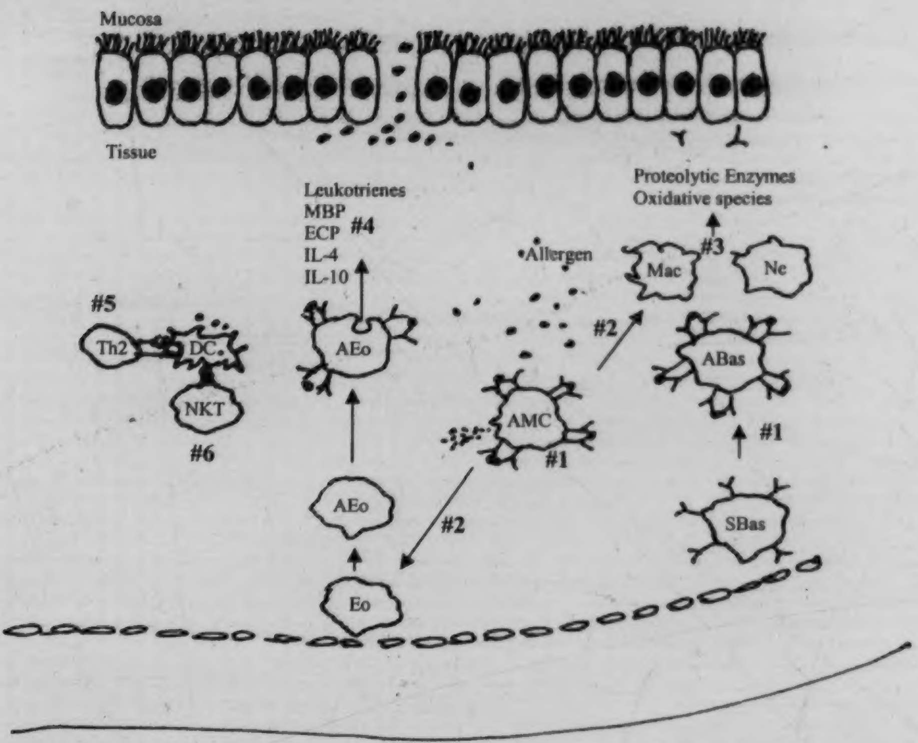

Figure 1.4. Late phase reaction of the effector phase of Type I Hypersensitivity. AMC = activated mast cell, SBas = sensitized basophil, ABas = activated basophil, Mac = macrophage, Ne = neutrophil, Eo = eosinophil, AEo = activated eosinophil, DC = dendritic cell, NKT = natural killer T cell

VII. Role of PAR-2 during the effector phase of type I reactions

i. Endogenous activators of PAR-2 during the effector phase

Mast cell tryptase

One of the major proteinases thought to activate PAR-2 is mast cell tryptase, a major secretory protein of human mast cells. Tryptase has been shown to activate PAR-2 on epithelial, endothelial cells, and on neurons (159, 168, 218-220). Because tryptase can activate PAR-2 it suggests that there is a predominant role for PAR-2 in humans under pathological circumstances where mast cells are involved. This includes during inflammation, type I hypersensitivity reactions, and wound repair (156, 221).

Tryptase is the most abundant proteinase in the granules of human mast cells. It makes up to 25% of the total cellular proteins and furthermore it is expressed by almost all of the subsets of human mast cells (222). There are distinct differences seen in the expression of tryptase in mast cells between species (223). Mast cell tryptase is quite abundant in the guinea pig (224), however, it is a less prominent proteinase in rat or murine mast cells. It has been previously shown that human tryptase purified from either human lung, skin, or mast cell lines, can cleave PAR-2 and induce signal transduction in transfected cells as well as many other cell types that naturally express PAR-2 at physiological levels (156, 159, 171, 218, 220, 225-229). Such signals appear to be mediated by tryptase, for they are abolished by selective inhibitors, and are PAR-2 dependent because they are absent from non-transfected cells and are diminished by selective down-regulation of PAR-2.

Importantly mast cell tryptase levels have been shown to be elevated in the lungs of asthmatic patients (230-232). Moreover, mast cell tryptase inhibitors can block allergen-induced AHR (233-235) and tryptase can have many pro-inflammatory effects in the airways (236-243). Furthermore, the hyperalgesic effects of tryptase in the skin are greatly diminished in PAR-2KO mice (244). A report in 2001 by Huang et al questioned the ability of mast cell tryptase to activate the PAR2 receptor (245). The exact explanation for this discrepancy has not been found. However, it is entirely possible that alterations in the extent of the receptor's glycosylation could dramatically affect the ability of mast cell tryptase to cleave and subsequently activate PAR-2 (246). Thus, de-glycosylation in vivo could regulate the tryptase activity as it pertains to the activation of PAR-2.

Trypsin

Proteinases other than mast cell tryptase may also have the ability to activate PAR-2 in the airways during the the effector stage of a type I hypersensitivity reaction. Trypsins, as well as trypsin-like proteinases are the most potent activators of PAR-2 (247). Trypsin has been shown to be expressed in mast cells and trypsin-like enzymes have been detected in airway epithelial cells (248) as well as in the airway secretions of asthmatic patients (249, 250). Thus, both trypsin and mast cell tryptase could have a combined effect and heighten PAR-2 activation during the effector stage.

Neutrophil proteinases

Neutrophil-derived serine proteinases, human leukocyte elastase, cathepsin G, and proteinase 3 are stored in the azurophilic granules of neutrophils as active enzymes. The major physiological function of the proteinases is commonly thought to be the intra-lysosomal degradation of engulfed cell debris or microorganisms (251, 252). Along with their roles in lysosomal degradation, these proteinases can play roles in extracellular proteolytic processes at sites of inflammation and have been shown to mediate at least some their effects through PAR-2. Uehara *et al* have recently shown that human leukocyte elastase, cathepsin G, and proteinase 3 are able to activate PAR-2 on oral epithelial cells, as well as on human gingival fibroblasts (253, 254). Furthermore, these authors have shown that PAR-2 activation of human gingival fibroblasts causes the release of the inflammatory cytokines IL-8 and MCP-1 (254).

ii. Effects of PAR-2 activation on resident lung cells

Several observations suggests that PAR-2 has an important role in airway inflammation during the effector phase of asthma. Firstly mast cell tryptase seems to play an important role in both airway inflammation and AHR (183). Second, PAR-2 is markedly up-regulated following exposure to pro-inflammatory stimuli or cytokines that play a role in chronic atopic asthma (255).

Epithelium

PAR-2 is highly expressed in the apical region of the airway epithelium (256). This suggests that the epithelium plays an active role during type I reactions such as asthma. Indeed, PAR-2 activation has been shown to stimulate the release of MMP-9 in a human airway epithelial cell line (257). This implicates PAR-2 activation on epithelial cells in the reorganization of lung extracellular matrix proteins during inflammatory responses. Others have shown that PAR-2 activation of airway epithelial cells releases eosinophil survival-promoting factors such as GM-CSF (258). Moreover, in human respiratory epithelial cells, PAR-2 activation stimulates release of IL-6, IL-8, and PGE_2 (259).

Smooth muscle cells

Studies have demonstrated PAR-2 immunoreactivity in the smooth muscle surrounding the endothelium of bronchial vessels (260). Interestingly, PAR-2 agonists are capable of causing bronchoconstriction (236). Whether this response is direct or dependent on secondary effects such as the generation of bronchoactive peptides and/or other mediators remains to be determined. However, along with its bronchoconstrictive properties studies have also suggested that PAR-2 is able to produce a relaxant effect in isolated main bronchi that may have a protective effect in the airways (248). Nitric oxide (NO) and prostanoids like PGE_2 may be involved in this process, because potentiation of PAR_2-induced bronchoconstriction has been observed upon inhibiting NO synthase or after prostanoid generation and COX-2 activation (261). In addition to a direct effect on bronchial smooth muscle, PAR-2 activation can act as an enhancer of histamine-mediated and leukotriene-mediated smooth muscle contraction (262). Thus, there is strong evidence that PAR-2 activation can promote both contractile and dilator mechanisms in the airways.

Endothelium/vasculature

In the vasculature, PAR-2 has many effects that are pro-inflammatory. Activation of PAR-2 increases vascular permeability when specific PAR-2AP are injected into the hindpaws of rats (263, 264). This increase in vascular permeability allows leukocytes to move readily into inflammed tissue. Moreover, activators of

PAR-2 can relax rings of rat aorta or porcine coronary artery. Relaxation is dependent on endothelial NO synthase activity (163, 165, 167). In contrast, trypsin stimulates contraction of rabbit aorta in the absence of endothelium (265). Moreover, i.v. injection in rats of PAR-2AP decreases blood pressure, consistent with the release of NO from endothelial cells (165). Furthermore, PAR-2 agonists increase IL-6, induce von Willebrand factor release, and are mitogenic for human umbilical vein endothelial cells (HUVEC) (162, 266-268). Interestingly, PAR-2 is up-regulated in HUVEC cells following their stimulation with inflammatory mediators such as TNF (269). This could be of importance in mediating inflammation during the effector stage of type I hypersensitivity reactions.

iii. Immune cells recruited to the airways during the effector phase

PAR-2 is expressed by various cells involved in the effector phase of type I responses, including T cells, mast cells, eosinophils, and neutrophils (166, 169, 270-272). Evidence points to a regulatory role of PAR-2 in leukocyte functions.

T cells

Several T cell lines express PAR-2 (160). Furthermore, trypsin, or PAR-2AP, can induce Ca^{2+} mobilization in Jurkat and HPB.ALL T cell lines (166) and may be important in activation of T cells, as PAR-2 activation is involved in Vav1-mediated signaling (273).

Mast cells

The localization of PAR-2 on human mast cells was first reported by D'Andrea *et al.* (270). Interestingly, PAR-2 was localized not only on the membrane of mast cells, but also on the membrane of the intracellular tryptase-positive granules

(270). Moreover, rat peritoneal mast cells also express PAR-2 mRNA (274). The precise role of PAR-2 on and in mast cells, especially with regard to a potential autocrine regulatory mechanism via tryptase activation, requires additional investigation. However it was recently shown that the Th1 cytokine IL-12 can down-regulate expression of PAR-2 while the Th2 cytokine IL-4 can up-regulate the receptor (275).

Neutrophils

Trypsin and PAR-2AP are also able to activate PAR-2 expressed on human neutrophils (169). This PAR-2 activation can cause changes in cell shape as well as the up-regulation of CD11b/CD18. In co-culture of human neutrophils and endothelial cells, PAR-2 agonists induced L-selectin shedding and CD11b/CD18 up-regulation in neutrophils (156). Moreover, PAR-2 agonists can induce Ca^{2+} mobilization in human neutrophils and enhance neutrophil motility in 3-D collagen gel lattices (276). Finally, PAR-2 activation on neutrophils promotes IL-8 and lactoferrin release (277).

iv. Importance of PAR-2 in allergic inflammation in the airways

Direct evidence for involvement of PAR-2 in allergic inflammation of the airways has been discovered (278). To study the importance of PAR-2 in airway inflammation, PAR-2KO mice and mice over-expressing human PAR-2 (PAR-2TR) were used. This over-expression of PAR-2 mimicked the fact that PAR-2 is up-regulated by inflammatory agents during type I reactions in the lung. Sensitization and challenge of wild-type mice led to infiltration of inflammatory immune cells, mostly eosinophils, into the lumen of the airways and also induced AHR.

Interestingly, both eosinophilic airway inflammation and AHR were increased in PAR-2TR mice, while this inflammation and AHR was markedly diminished in PAR-2KO animals. Additionally, in PAR-2KO mice the IgE response to OVA was strongly reduced, implicating PAR-2 in immune responses.

These results indicate that PAR-2 is important in asthmatic type I reactions. PAR-2 is also important in the pathology of atopic dermatitis, another allergic disease.

v. Role of PAR-2 in atopic dermatitis

Atopic dermatitis is the most common type I hypersensitivity reaction that affects the skin. In murine models of experimentally induced allergic dermatitis, a pro-inflammatory role of PAR-2 has been demonstrated (279, 280). In one model stimulation of PAR-2 resulted in an increase in ear swelling (280). Others examined the underlying mechanisms responsible for the PAR-2 induced effects during atopic dermatitis using PAR-2KO mice (279). They found that PAR-2 is involved in edema formation, plasma extravasation, up-regulation of cytokines (IL-6), cell adhesion molecules (ICAM-1), and selectins (E-selectin). Furthermore, intravital microscopy studies showed that velocity and adhesion of leukocytes is impaired in PAR-2KO mice compared with wild-type controls. Finally, *ex vivo* studies in skin biopsies of human volunteers have confirmed a role for PAR-2 in selectin regulation of dermal blood vessels (279), as PAR-2 agonists induced a marked increase of E-selectin immunoreactivity in dermal endothelial cells in comparison to control tissues. Together, these results support a pro-inflammatory role of PAR-2 in atopic dermatitis.

Thus, PAR-2 may be important in both the sensitization phase as well as the effector phase in type I hypersensitivity reactions such as asthma. As it relates to the sensitization phase, PAR-2 activation could be pushing the immune system towards atopic type I sensitization and away from a protective immune response named "respiratory mucosal immune tolerance".

VIII. Respiratory mucosal immune tolerance

The respiratory mucosa is the first surface of the body that interacts with inhaled antigens. The immune system must be able to discern antigens that are innocuous from those that are pathogenic. Normally innocuous proteins that challenge the immune system by the respiratory route provoke mucosal immune tolerance. Respiratory mucosal immune tolerance was first described by Holt et al. over 25 years ago (281).

In the 1970s there was a great increase in knowledge of the control mechanisms that regulate IgE synthesis in experimental animals. However, these studies used adjuvant dependant immunizations regimes to skew the model towards Th2 IgE immune responses. The most favorable of these immunizations, or the ones that provided the strongest production of IgE, were those involving the protein antigen with $Al(OH)_3$ injected i.p.. Holt et al explored a more natural route in developing these allergic responses and thus gave the protein antigen via the respiratory route as they postulated that mice would preferentially default to the IgE subtype and subsequently develop allergic reactions. However, they found that by giving the antigen into the respiratory tract it provided protection towards the development of allergic responses in the airways (281).

In these studies mice were exposed to aerosolized OVA intranasally (i.n.). These mice had transient IgE responses that declined by the 7th week post exposure. Although IgE responses were low, mice displayed a strong haemagglutininating antibody (HA) response indicating that Abs of a different isotype were being produced against the OVA. Furthermore, when these animals were exposed to OVA and $Al(OH)_3$ i.p. immunization their IgE Ab responses were suppressed. This appeared to show a protective effect towards the strong Th2 polarizing immunization. The protective effect was also seen in naïve mice that had splenocytes adoptively transferred into them from mice repeatedly exposed to OVA. These results indicated the existence of suppressor cells that specifically inhibit the development of allergic responses.

It has been found that these suppressor cells $CD4^+$ $T_{reg}1$ or Th3 cells that are generated by interactions with conventional "modulated lung DCs" and plasmacytoid DCs (pDCs).

i. Cells involved in the development of respiratory mucosal immune tolerance conventional "modulated DCs"

The characteristics and roles of DCs in the sensitization phase of type I hypersensitivity reactions have been discussed above. DCs direct immune responses by their ability to direct T cell functions. These functions include the production of cytokines that induce specific immune responses. In the case of respiratory mucosal immune tolerance these DCs become "modulated". A "modulated DC" (modDC) is a DC that acquires an innocuous foreign protein antigen, such as OVA, in the absence of a "danger signal" but in the presence of certain immunosuppressive molecules that constitute a "tolerogenic signal" (282). IL-10 is one mediator that is thought to provide "tolerogenic signals" to immature DCs to activate them to become modDCs (282). These modDCs have relatively high amounts of co-stimulatory molecules as well as MHC II on them when compared to immature DC. Therefore, when these modDCs interact with naïve Th0 cells, the Th0 cell is not anergized, as in the case of immature DCs, but produce and expand $T_{reg}1$ or Th3 subtypes (282-287). The exact subtype created depends on the cytokines secreted by the modDC when it engages the Th0 cell. If they produce IL-10 and IFNα then $T_{reg}1$ are produced, but if the modDCs produce IL-10 and IL-4, as is the case when OVA is administered into the lungs, then Th3 cells are produced (285-287). Along with modDCs, pDCs are also needed for the development of airway mucosal immune tolerance.

Plasmacytoid Dendritic cells (pDCs)

Originally called "T-associated plasma cells" because they are found in T cell zones in lymphoid tissues (288), pDC are recognized to have many functions and roles in immune responses. In the development of mucosal immune tolerance it is thought that pDCs are responsible for preventing the development of $CD4^+$ Th2 cells by promoting the development of $T_{reg}1$ or Th3 cells, while modDCs are important for generating T cell division and priming of Th0 cells into $T_{reg}1$ or Th3 cells (289). Thus, the development of mucosal immune tolerance needs several cell types to orchestrate the immunological outcome.

$CD4^+$ $T_{reg}1$ and Th3 cells

$T_{reg}1$ cells do not express detectable levels of the T_{reg} marker GITR, but do express low levels of CTLA-4 and CD25 (284-287). Th3 cells on the other hand have low levels of CD25 and moderate levels of GITR and CTLA-4. Both $T_{reg}1$ and Th3 cells exert their immunosuppressive effects by the secretion of specific cytokines. $T_{reg}1$ cells secrete IL-10 and low levels of TGFβ, while Th3 cells produce IL-10 and TGFβ (286, 287, 290).

ii. Important cytokines in the development of mucosal immune tolerance

IL-10

IL-10 is secreted by activated monocytes, macrophages, Th2 cells, T_{reg} cells, $T_{reg}1$ cells, Th3 cells, as well as mast cells (291). IL-10 has roles in either innate and adaptive immunity that are immunosuppressive or immunostimulatory. One of the essential roles of IL-10 is to limit inflammatory responses by targeting macrophages, neutrophils, mast cells, and eosinophils (291). IL-10 inhibits the transcription of important genes involved in the inflammatory response (291-293). In particular IL-1, IL-6, IL-8, and IL-12 are downregulated (292, 293). Indeed, IL-10 is protective against the large cytokine release occurring during endotoxic shock (291). Furthermore, IL-10 can inhibit microbe killing by macrophages and down-regulate $CD4^+$ T cell responses (291). On the stimulatory front low levels of IL-10 promote Th2 responses by inhibiting secretion of IFNγ and IL-2 by $CD4^+$ Th1 cells, as well as up-regulation of MHC II molecules and Igs in B cells (294, 295).

iii. Molecular mechanisms of mucosal immune tolerance (Fig. 1.5)

Respiratory mucosal immune tolerance begins with the inhalation of an innocuous protein antigen. This inhaled antigen produces a "tolerogenic signal" in the airways when taken up by immature DCs (Fig. 1.5 #1). This "tolerogenic signal" influences DCs to differentiate into modDCs (moDC), and present the antigen on

MHC II complexes when they migrate to the draining LNs (Fig. 1.5 #2). In the LNs the modDC interacts not only with naïve $CD4^+$ Th0 cells but also pDCs. The modDC stimulates T cell division and development of the $CD4^+$ Th0 cells into $T_{reg}1$ or Th3 cells that appears to involve ICOS-ICOSL signaling (296), while the pDCs silence any Th2 responses that may arise from the modDC (284-287, 289, 290) (Fig. 1.5 #3). With OVA, a common innocuous protein antigen administered into the airways to induce mucosal immune tolerance in mice, Th3 cells develop as the modDCs in the LNs and secrete IL-10 and IL-4 (286). Once they are generated, $T_{reg}1$ or Th3 cells home to the tissues where the immature DCs first received the "tolerogenic signal" (Fig. 1.5 #4). In the respiratory tract the immune tolerant tissue is the lung. Once in the tissue the $T_{reg}1$ or Th3 cells can be activated by the same antigen to down-regulate any pathogenic immune response, such as asthma (284, 285, 287, 290) (Fig. 1.5 #5). Therefore, for a protein antigen to become an allergen it must push the immune response away from a tolerogenic one towards atopic/allergic sensitization. The protein antigen must create a "danger signal" in the mucosa (airway mucosa) that is able to push the immune system towards an atopic/allergic Th2 response. This atopic/allergic "danger signal" then overrides the "tolerogenic signal" and promotes allergic sensitization. As many potent allergens have intrinsic serine proteinase activity or are inhaled in particles that are rich in serine proteinases this atopic/allergic "danger signal" could be the result of mediators released as a result of PAR-2 activation.

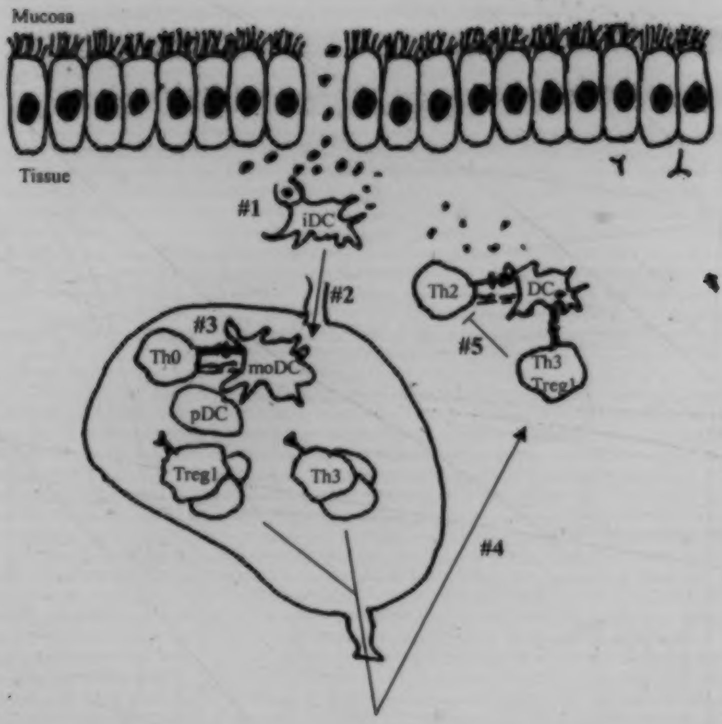

Figure 1.5. Cellular mechanisms of mucosal immune tolerance. Th0 = naïve T cell, iDC = immature DC, moDC = modulated DC, pDC = plasmacytoid DC, $T_{reg}1$ = T-regulatory-like-1 cell, Th3 = T-helper-3 cell

IX. Studying the role of PAR-2 in allergic sensitization and allergic inflammation using *in vivo* mouse models

The use of *in vivo* models to study immunological mechanisms has the major advantage over *in vitro* systems as they are complete biological systems. Because of this, observations seen using these models provide greater relevance when relating them back to human conditions. Therefore to study the role of PAR-2 in allergic sensitization and allergic airway disease we chose to use the animal models outlined below. We have chosen to use mouse models to study PAR-2 activation in allergic sensitization and allergic inflammation. The reason for this is that there has been a PAR-2KO mouse developed that we intend to use to address specific mechanisms seen in our models.

i. Role of PAR-2 in the development of allergic sensitization

When OVA is administered into the airways of mice, the resulting immune response is mucosal immune tolerance (153, 281, 286). In some of these models OVA is administered over a short, three consecutive day, period (286). In other models more administrations of antigens are given over a longer period (5 times once every 5th day) (153). In both cases immunological tolerance results that protects the animals from the development of allergic responses (AHR and airway inflammation) even when the animals are subsequently immunized to OVA in the presence of a strong Th2 adjuvant such as $Al(OH)_3$.

Using these models we administered OVA with a PAR-2AP to mimic a potential allergen that has proteinase activity or is inhaled in particles rich in proteinases. Using this system we mimicked the PAR-2-activating potential of an allergen with serine proteinase activity, but avoided the PAR-2-independent effects of proteolytic enzymes. Using these models we tested whether PAR-2 activation alone directed the resulting immune response away from mucosal immune tolerance towards allergic sensitization.

ii. Role of PAR-2 in the effector phase of atopic/allergic asthma

In mice, many antigens have been successfully used to induce allergic asthma. The most commonly used antigen is OVA (121, 297-299), but others include bovine serum albumin (300), sheep red blood cells (301), ß-lactoglobulin (302), schistosoma (303) and leishmania proteins (304-306), coagulated egg white implants (307), *Aspergillus fumigatus* (308), cockroach antigens (309), olive (310, 311), birch (312) and ragweed (313, 314) pollens, and house dust mite antigens (315-321). Additionally, allergic disease can be induced in naïve mice by adoptive transfer of fully activated and polarized Th2 cells from immunized wild-type (300, 322, 323) or DO11.10 (324-326) mice. The induction of allergic asthma in mice in response to non-allergenic proteins suggests that allergenicity is not a necessary feature when strong Th2 polarizing adjuvants such as $Al(OH)_3$ are used to prime the animals.

For our experiments studying the role of PAR-2 in the effector stage of allergic asthma we sensitized and challenged, the mice with OVA (using $Al(OH)_3$ adjuvant) as this is the same antigen we used during the sensitization experiments. Furthermore, this sensitization and challenge protocol shows pronounced signs of allergic airways inflammation, including eosinophilic inflammation, mucus hypersecretion, airway hyperresponsiveness (AHR), airway remodeling, and elevated production of IgE.

iii. Strain of mouse used

Interestingly, there is mouse strain variability in development of allergic inflammation, a feature that implicates genetic factors (319, 320, 327-334). For all of our experiments we used Balb/c because they have a "Th2 phenotype" or are predisposed to Th2 responses. This has much relevance to the development of allergic sensitization and responses as it likely mimics genetic components in some asthmatic patients.

iv. mouse models and human asthma

These models focused on the factors governing the allergic immune response, on modeling clinical behavior of allergic asthma, and led to insights into pulmonary pathophysiology. Although mouse models rarely completely reproduce all the features of human disease, after sensitization and respiratory tract challenges with antigen, wild-type mice develop pathology that resembles allergic asthma, characterized by eosinophilic lung inflammation, AHR, increased IgE, mucus hypersecretion, and eventually, airway remodeling (327-334). There are, however, differences between mouse and human physiology. Three examples of such differences relate to both clinical manifestations of disease and underlying pathogenesis. First, in contrast to patients who have increased methacholine-induced AHR even when they are symptom-free, mice exhibit only transient methacholine-induced AHR following allergen exposure. Second, chronic allergen exposure in patients leads to chronic allergic asthma, whereas repeated exposures in sensitized mice causes suppression of disease. Third, IgE and mast cells, in humans, mediate early and late phase allergic responses, though both are unnecessary for the generation of allergic asthma in some mouse models (330-332).

X. Hypothesis and conceptual models

There is much evidence that PAR-2 may be important in both the sensitization phase and the effector phase of the type I hypersensitivity reaction asthma. For the sensitization phase, the proteinase activity of many potent allergens suggests that PAR-2 could be recognizing the inhaled allergens and initiating danger signals that push the immune response towards atopy. For the effector phase both proteinases and PAR-2 are integral in the pathogenesis of not only asthma, but other atopic type I diseases such as dermatitis.

i. Role of PAR-2 in the development of atopic/allergic sensitization

Hypothesis

PAR-2 activation interferes with the development of respiratory mucosal immune tolerance and promotes the development of atopic/allergic sensitization by the release of specific inflammatory mediators.

Methodology

Mucosal exposure of mice to house dust mite allergens, which are able to activate PAR-2, leads to allergic sensitization, while mucosal exposure to OVA has been shown to lead to immune tolerance. To test our hypothesis we will use a murine system with mucosal exposure to OVA as an Ag, and to a PAR-2 activating peptide (PAR-2AP), to mimic the potential of the allergen or other inhaled proteinases to activate PAR-2. A scrambled peptide with the same amino acid composition as the PAR-2AP was used as a control (PAR-2CP). We will ensure that our OVA and PAR-2 peptides are LPS free as we do not want any TLR-4 activation that we could mistake as PAR-2 mediated effects. This is especially true in the sensitization experiments as low levels of LPS have been shown to promote it.

Conceptual model

OVA administered alone will cause the mice to develop respiratory mucosal immune tolerance and thus will be protected from the development of allergic disease in the airways even when given a strong Th2 polarizing immunization (Fig. 1.6). When the PAR-2AP is administered into the airways along with the OVA the PAR-2AP will bind to the PAR-2 receptors on specific cell types and cause the release of mediators. These mediators in turn will provide a "danger signal" that will result in the animal's allergic sensitization towards the OVA and the animal will develop asthmatic symptoms (airway inflammation and AHR) (Fig. 1.7).

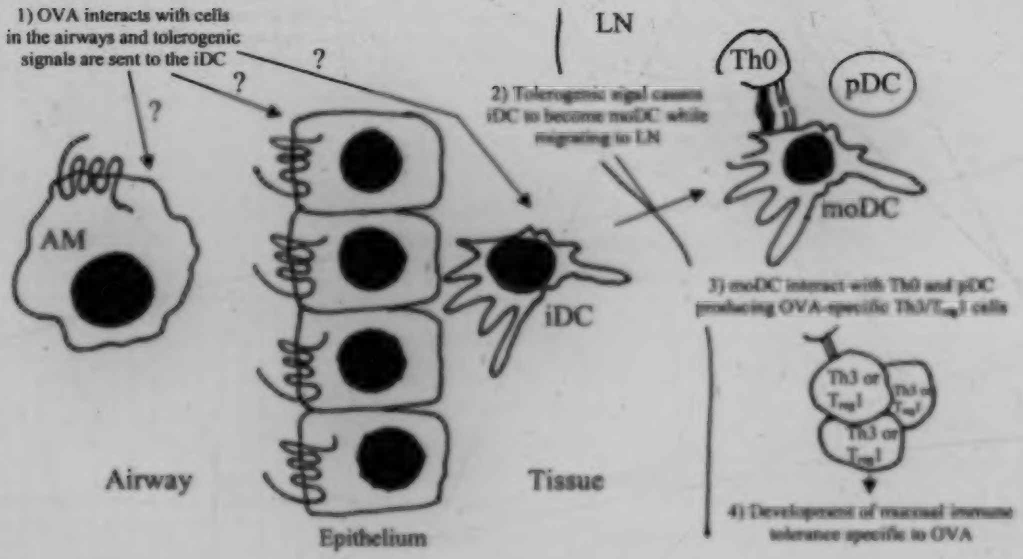
1) OVA interacts with cells in the airways and tolerogenic signals are sent to the iDC
?
?
?
LN
Th0
pDC
2) Tolerogenic signal causes iDC to become moDC while migrating to LN
moDC
AM
iDC
3) moDC interact with Th0 and pDC producing OVA-specific Th3/$T_{reg}1$ cells
Th3 or $T_{reg}1$
Th3 or $T_{reg}1$
Th3 or $T_{reg}1$
Airway
Tissue
Epithelium
4) Development of mucosal immune tolerance specific to OVA

Figure 1.6. OVA administered into the airways promotes mucosal immune tolerance

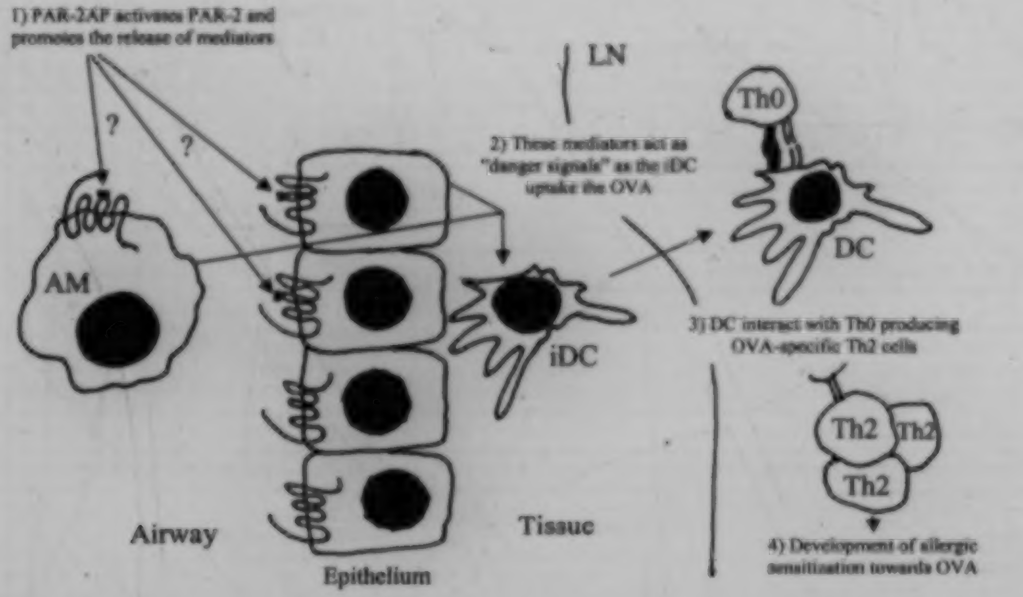
1) PAR-2AP activates PAR-2 and promotes the release of mediators
LN
?
?
Th0
2) These mediators act as "danger signals" as the iDC uptake the OVA
DC
AM
iDC
3) DC interact with Th0 producing OVA-specific Th2 cells
Th2
Th2
Th2
Airway
Tissue
Epithelium
4) Development of allergic sensitization towards OVA

Figure 1.7. PAR-2 activation interferes with the development of mucosal immune tolerance and promotes allergic sensitization.

ii. Role of PAR-2 in the effector phase of atopic/allergic asthma

Hypothesis

Enhanced PAR-2 activation in the airways during allergen challenge in sensitized mice will lead to an exacerbation of the pathological features of asthma, eosinophilic airway inflammation and AHR.

Methodology

Mice were sensitized to OVA using an intraperitoneal (i.p.) injection of OVA + $Al(OH)_3$. All mice were subsequently challenged with OVA alone or with PAR-2AP to activate PAR-2 in the airways. A scrambled version of the activating peptide served as a control (PAR-2CP). Airway inflammation and AHR were measured in the two groups and differences noted.

Conceptual model

When the OVA sensitized mice are given OVA in the airways this will result in the release of pro-inflammatory mediators as well as endogenous proteinases in the mucosa that mediate the development of asthmatic like responses. When PAR-2AP is given with OVA, it together with endogenous proteinases released will cause widespread activation of PAR-2 in the airways/mucosa enhance cytokine release. This enhanced cytokine release will in turn enhance the pathological features of asthma, eosinophilic airway/lung inflammation and AHR.

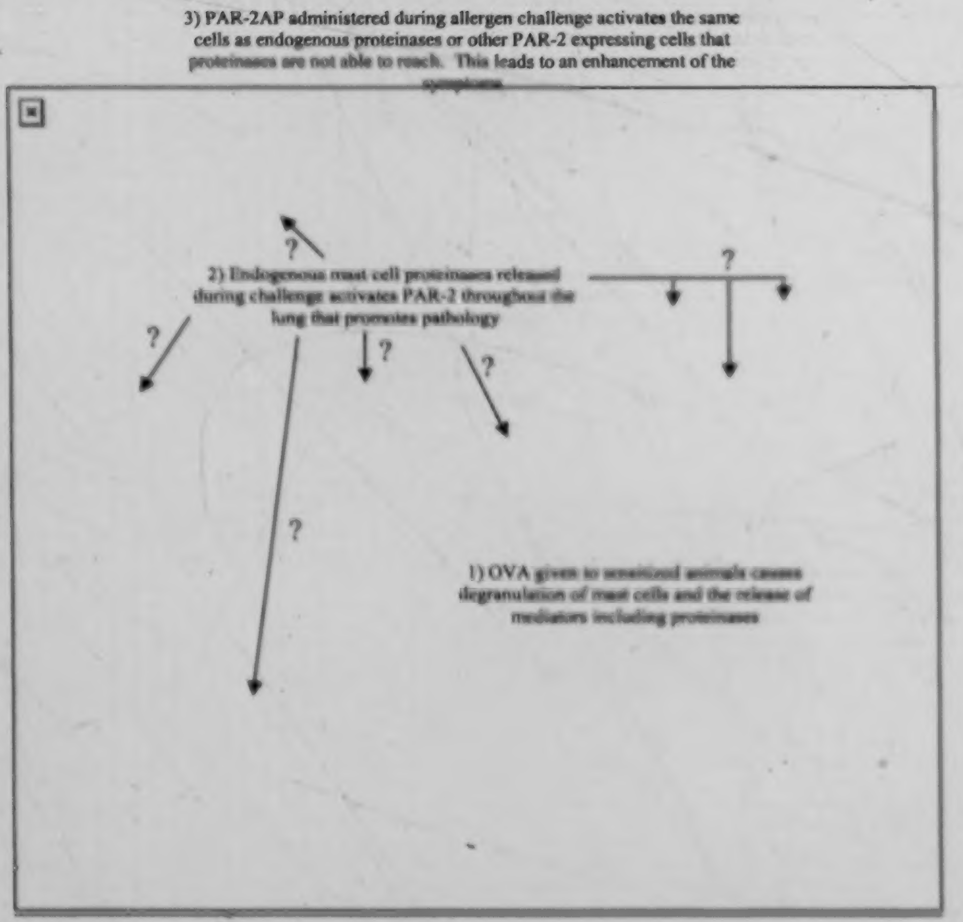
3) PAR-2AP administered during allergen challenge activates the same cells as endogenous proteinases or other PAR-2 expressing cells that proteinases are not able to reach. This leads to an enhancement of the symptoms
?
?
2) Endogenous mast cell proteinases released during challenge activates PAR-2 throughout the lung that promotes pathology
?
?
?
?
1) OVA given to sensitized animals causes degranulation of mast cells and the release of mediators including proteinases

Figure 1.8. Conceptual model: PAR-2 activation during allergen challenge enhances pathological features of asthma. AMC = activated mast cell, SBas = sensitized basophil, ABas = activated basophil, Mac = macrophage, Ne = neutrophil, Eo = eosinophil, AEo = activated eosinophil, DC = dendritic cell, NKT = natural killer T cell

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Chapter 2: Results section #1

PAR-2 expression in murine lung and nasal mucosa: effects of PAR-2 activation in the airways using *in vivo* administration of PAR-2AP in these mice.

Most of the data presented here have been published in:

Ebeling, C., P. Forsythe, J. Ng, J. R. Gordon, M. Hollenberg, and H. Vliagoftis. 2005. Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. *J Allergy Clin Immunol* 115:623-630.

All experiments were designed and analysized by Ebeling, C and H. Vliagoftis

M. Hollenberg – provided the PAR-2AP and CPs for our experiments

I. Introduction

Although the expression on human cells is known, less is known about its expression in the airways of mice. As we administered PAR-2AP and OVA via the intranasal (i.n.) route, in the following chapters, it was important that we identified the distribution of PAR-2 expression in the lung and in the nose.

We hypothesized that PAR-2 expression in the lung would mirror that seen in the human and be present on epithelial, endothelial, smooth muscle cells, and AM.

Part of our hypothesis was that PAR-2 activation results in release of mediators that cause allergic sensitization to OVA and increase the pathology of allergic airway inflammation. Therefore, we assessed cytokine mediators at the mRNA level, or as protein released following administration of PAR-2AP into the airways. Cytokine release could promote leukocyte inflammation or AHR. As PAR-2 is vital for many inflammatory responses PAR-2 activation alone could be sufficient to cause responses in the lung that manifests asthma-like responses in the absence of allergens.

II. Methods

Animals

Male Balb/c mice (18-20g) were purchased from Charles River Laboratories (St.Constant, Quebec, Canada). The mice were housed and experimented in Virus and Antibody Free conditions and maintained on a 12 h light-dark schedule. The University of Alberta Health Sciences Laboratory Animal Ethics Board (Edmonton, AB, Canada) approved all experiments described in this study.

PAR-2 localization in tissue sections by immunohistochemistry

Mice were sacrificed and lungs were excised and placed in Histo Prep™ (Fisher Scientific, Ottawa, ON, Canada) and quick frozen in super-cooled methanol. Frozen sections (5 μm) were prepared and placed in cold acetone (20°C) for 10 min, washed in PBS (5 min), placed in methanol containing 0.3% H_2O_2 for 35 min to block endogenous peroxidases then incubated with the anti-PAR-2 B5 Ab (1/250 dil in 100 μl PBS) or anti-PAR-2 B5 Ab preabsorbed with immunizing peptide (65 μg/ml in 100 μl of PBS for 1 h at 4°C) for 4 h at room temp. Sections were then washed in PBS (5 min), incubated with HRP labeled $F(ab')_2$ goat anti-rabbit IgG (1/2000 dil in 100 μl PBS; Jackson ImmunoResearch Inc., West Grove, PA, USA) for 1 h at room temp, washed in PBS (5 min) then stained with diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA, USA). Sections were then rinsed in water (5 min), counterstained with Harris' Hematoxylin, and dehydrated in increasing ethanol/xylene gradient. Slides were photographed using a Nikon DXM 1200 digital camera mounted on a Nikon E 800 microscope (Nikon Corporation, Tokyo, Japan).

PAR-2 localization in BAL cells by immunohistochemistry

Cytospins of BAL cells on slides were subject to the same staining protocol as the tissue sections with the exception that slides were incubated with the primary B5 Ab (or preabsorbed B5 Ab) at 1/100 dil for 2.5 h at room temp while the secondary $F(ab')_2$ fragment was incubated at 1/1000 dil for 1.5 h at room temperature.

Intranasal administration of PAR-2AP in mice

Following light anesthesia with ketamine (75 mg/kg) and acepromazine (6µg/kg), some groups of mice were administered on day 12 alone or on days 12 and 14 i.n., a PAR-2AP (SLIGRL-NH_2, 25 µL of 100 µM solution in saline) while other mice received a PAR-2 control peptide PAR-2CP (LSIGRL-NH_2, 25 µL of 100 µM solution in saline). Mice were held with their heads pointing towards the ceiling for 30 s following administration of the peptides.

Bronchoalveolar lavage (BAL)

All BALs were taken 24 h following the final administrations of PAR-2AP or CP, mice were sacrificed (ketamine, 0.5g/kg; xylazine, 0.1g/kg, i.p.) trachea exposed and cannulated and lungs rinsed with PBS (7 × 0.8 mL) along with chest cavity massage. 25µL of lavage fluid was diluted ½ with trypan blue and the numbers of total cells in BAL were determined using a hemocytometer. Differential cell counts of BAL were determined by staining 5×10^3 cytospun cells using Diff-Quick methodology.

Airway hyperresponsiveness (AHR)

All AHR measurements were taken 24 h following the final administrations of PAR-2AP or CP, enhanced pause (Penh) was measured in mice using whole-body plethysmography to determine bronchoconstriction in response to methacholine (MeCh) challenge. Conscious mice were placed in chamber and allowed to normalize for a period of 10 min. Mice were then exposed to saline aerosol (MeCh vehicle) (3 min) and average Penh values were recorded over a 5 min period to determine baseline reactivity. Mice were then challenged using increasing concentrations of MeCh (2, 4, 8, 16, 32 μg/mL) via ultrasonic aerosol (3 min) and the average Penh values were recorded over a 5 min period following challenge. A 10 min rest period followed each 5 min Penh recording period.

Inflammatory cytokine mRNA analysis

Lungs from mice given the PAR-2AP or PAR-2CP were excised 4 h following i.n. administration on day 12 and total mRNA was isolated using Stat-60 reagent. mRNA (20μg) was lyophilized and kept at -70 °C until analyzed by RNase protection assay. Multi-Probe RNase Protection Assay was performed as per manufacturer's instructions using RNA probes synthesized from RiboQuant™ (BD Pharmingen, Mississauga, ON, Canada) template plasmids (TNF, IL-4, IL-5, IL-10, IL-13, MIP-1α, MIP-2, MCP-1, IL-6, IFNγ, and the two housekeeping genes L32 and GAPDH). Densitometry was performed on the signals for all genes and quantification was determined against the L32 housekeeping gene using AlphaImager software.

Cytokine assays on BAL fluid

IL-4, IL-5, IL-9, IL-10, IL-13, TNF, and IFN-γ were measured in BAL fluid samples to determine levels of inflammatory cytokines in the airway by ELISA as previously described (1). It must be pointed out that all ELISA measurements were conducted at the University of Saskatchewan by Dr. John Gordon's research technician.

Statistical analysis

Statistical differences in the means between treatment groups were determined by using a one-way ANOVA. The paired Student *t* test was used to compare the means of 2 groups. Differences in AHR were determined by *F* test analysis that compared values over the entire curves of each treatment group to one another. From this *F* score, a *P* value was generated. In all cases, a *P* value <0.05 was considered statistically significant.

III. Results

i. Localization of PAR-2 in the airways/lungs of Balb/c mice

PAR-2 was localized to the airway epithelium, endothelium, airway smooth muscle, and AM of OVA sensitized mice (Fig. 2.1A and 2.1B). PAR-2 was also abundantly expressed on the epithelium of the nasal cavity (Fig. 2.1C). Preabsorbtion of the B5 Ab with the immunizing peptide given to the rabbits to generate the B5 Ab abolished staining in all cases verifying the specificity of the results (Fig. 2.1D, 2.1E and 2.1F).

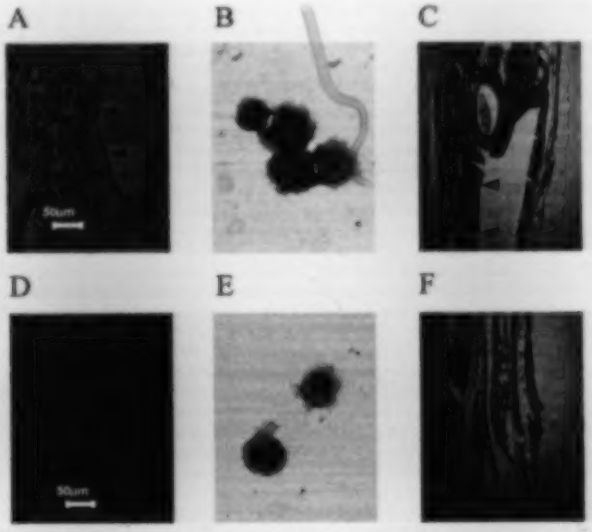

Figure 2.1. PAR-2 localization in/on the (A) lung tissue, (B) AM, and (C) nasal tissue of Balb/c mice. PAR-2 was localized to the airway epithelium (red arrows), smooth muscle (green arrows), and vascular endothelium (black arrows). As a negative control B5 Ab was preabsorbed with immunizing peptide (D, E, and F).

ii. Cytokine and chemokine mRNA expression in the lung following administration of PAR-2AP or PAR-2CP

After seeing PAR-2 expressed in the lungs of the mice, we studied the effects of its activation on the expression of inflammatory cytokines and chemokines at the mRNA level. Mice were given either the PAR-2AP or the PAR-2CP. Four h later mice were sacrificed, lung excised and total lung mRNA was isolated and screened using RNase protection assay for a panel of cytokines and chemokines. PAR-2AP alone up-regulated TNF and MIP-2 and to a smaller extent IL-13 mRNA levels in the lungs of sensitized mice 4h after administration. PAR-2CP showed no such effect (Fig. 2.2).

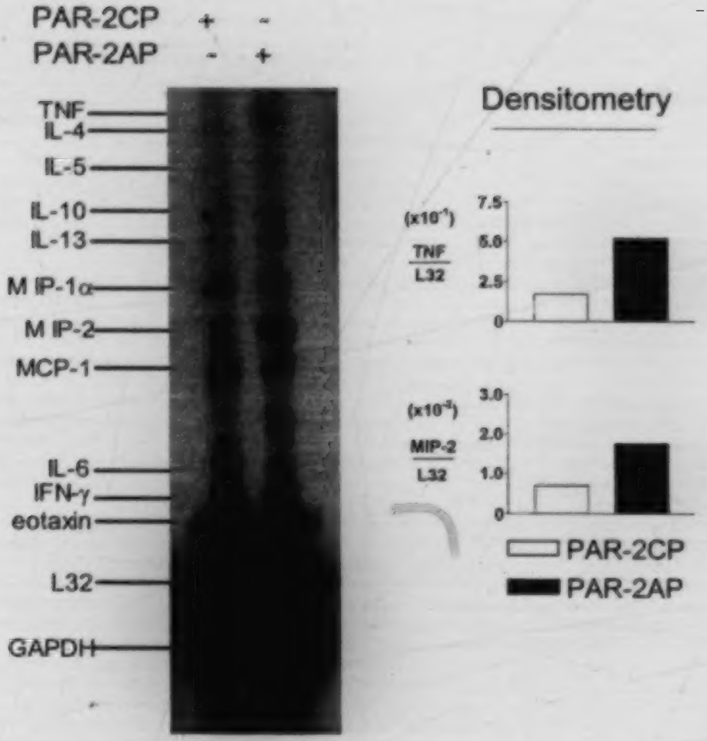

Figure 2.2. RNase protection assay for inflammatory cytokines present in the lung 4 h following administration of PAR-2CP or PAR-2AP to Balb/c mice and densitometry graphs of up-regulated genes (one representative experiment of two is shown. RNase protection assay was performed using mRNA isolated from one mouse given PAR-2CP or AP per experiment.

iii. Cytokine protein expression in the lung following administration of PAR-2AP or PAR-2CP

To assess inflammatory cytokines and chemokines released into the airways, mice were given either PAR-2AP or PAR-2CP and 24 h later the BAL fluid was taken from these animals and the levels of IL-4, IL-5, IL-9, IL-10, IL-13, TNF, and IFN-γ were determined by ELISA. Administration of PAR-2AP alone increased the levels of IL-13 in BAL fluid compared to PAR-2CP (53.6 ± 13.3 pg/ml and undetectable levels, respectively, n = 3, $p<0.05$), and the levels of TNF (72.2 ± 28.2 vs. 19.5 ± 19.5 pg/ml, n = 3, $p<0.05$) (Fig. 2.3). In all cases IL-4, IL-9, IL-10 and IFN-γ were not significantly altered, while IL-5 showed an upward trend but did not reach statistical significance.

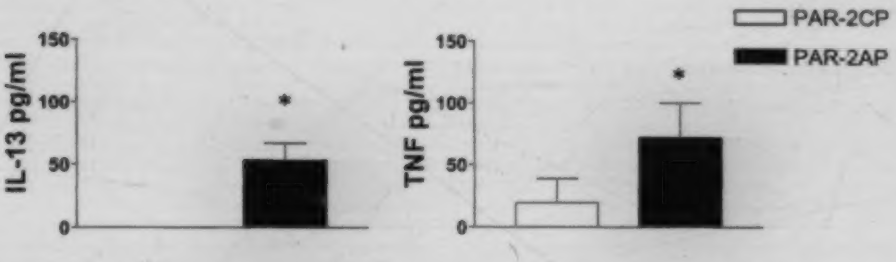

Figure 2.3. IL-13 and TNF levels in BAL fluid of sensitized mice treated with PAR-2CP or PAR-2AP. Results are shown as mean ± SEM (n=3; cytokine analysis in BAL of 3 separate mice administered PAR-2AP or PAR-2CP). *$p<0.05$ compared with PAR-2CP treated animals.

iv. Effects of PAR-2 activation on the development of cellular airway inflammation and AHR

We observed that administration of PAR-2AP alone induced up-regulation of inflammatory cytokines/chemokines that are integral in the development of inflammation (TNF) (2-4) as well as AHR (IL-13) (5,6). As a result we assessed whether or not PAR-2 activation alone would mediate the development of both of these pathogenic conditions. Mice were challenged twice on alternating days (12 and 14) and 24 h later mice were assessed for cellular inflammation, by BAL, and AHR by whole-body plethysmography. In both the PAR-2AP and PAR-2CP groups the cells seen in the BAL were exclusively alveolar macrophages. Furthermore, there were no differences in total cell numbers between the two groups. AHR was also not induced in any of the treatment groups (Fig. 2.4).

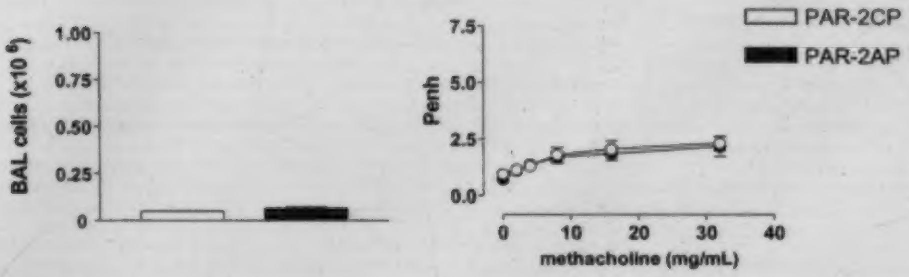

Figure 2.4. BAL total cell numbers as well as Penh values, from increasing doses of MeCh, in mice administered PAR-2AP or PAR-2CP alone (n=5 independent mice per group performed in 2 experiments). Results are shown as mean ± SEM.

Together these results indicate that PAR-2 activation by our PAR-2AP promotes the release of cytokines in the murine airways. However, PAR-2 activation alone was not sufficient to induce inflammatory cell infiltration or AHR in the airways.

IV. Discussion

We studied the localization of PAR-2 as well as the effects of its activation on mice. We chose to use a synthetic peptide to activate PAR-2 in the airways. This PAR-2AP is specific for PAR-2, as has been determined *in vivo* through the use of PAR-2 KO mice (7-9). We activated PAR-2 with 2.5 nmoles of PAR-2AP. This amount is similar to that used in other *in vivo* studies (10).

We found that PAR-2 was localized to the airway epithelium, smooth muscle, endothelium, and AM in the lungs mice. When administered into the airways the PAR-2AP was able to cause cytokine release. These cytokines however, did not cause cellular airway inflammation or AHR.

Although this release of these cytokines may not be strong enough to cause pathology in the lung it may have effects on the development of allergic sensitization to protein antigens that are inhaled concurrently with PAR-2 activation.

AM are attractive targets for study because they are situated throughout the airways and are one of the first cell types encountered by PAR-2 activating proteinases. Furthermore, AM can produce large amounts of TNF (11). TNF can activate DCs (12-14) directly (15), or indirectly by inducing the release of other mediators from the epithelium (16). As I have discussed in the introduction, one such mediator, GM-CSF, can activate DCs (17) and induce allergic sensitization to OVA when administered directly or as transgene expression in the airways (18). TNF release following PAR-2 activation could therefore have potent immunogenic properties and be an innate immune response initiated by PAR-2 activating allergens. This innate response could mediate adaptive Th2 immune response towards these allergens. When re-encountered in the respiratory tract these allergens could subsequently cause allergic reactions in the lung.

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Chapter 3: Results section #2

Chapter 2: The role of PAR-2 activation in sensitization to inhaled protein antigen, that under normal circumstances causes tolerance, and determination of whether TNF is integral in this sensitization process.

Adapted from:

Ebeling, C., T. Lam, J. R. Gordon, MD. Hollenberg, and H. Vliagoftis. 2007. Proteinase-activated receptor-2 promotes allergic sensitization to an inhaled antigen through a TNF-mediated pathway. *J Immunol* 179:2910-7.

All experiments were designed and analysized by Ebeling, C, T. Lam and H. Vliagoftis

T. Lam – did the early initial experiment seen in Figure 3.9A that Ebeling, C. reproduced and expanded upon

J. R. Gordon – measured OVA-specific Igs and T cell cytokines

MD. Hollenberg – provided the PAR-2APs and CPs

All of the authors read and approved the final manuscript

I. Introduction

As previously mentioned atopy may be defined as a genetically and environmentally determined predisposition to the development of disorders such as allergic rhinitis, atopic dermatitis or eczema, and allergic asthma (1,2). Atopy has increased dramatically in western societies over recent decades. While theories such as the hygiene hypothesis try to explain this increased prevalence, the main question remains unanswered: why and how do people develop atopy?

Many protein antigens we encounter through the respiratory tract promote mucosal tolerance that provides protective effects when we re-encounter the same protein. However, there are a limited number of inhaled antigens that become "allergens". The question is raised how or what links responses to these "allergens", when they are first encountered, to development of adaptive Th2 responses towards them.

For the past decade attempt to characterize the linkage between innate and adaptive immunity have been dominated by the TLR paradigm. Although this paradigm has been very effective in explaining Th1 adaptive immune responses towards pathogens, it has not explained why and how we develop Th2 responses towards many of the potent allergens we encounter in the environment. There have been no specific motifs in allergens that are recognized by any TLRs identified to date. Thus, another receptor system may be recognizing these allergens and promoting innate immune responses that result in Th2 adaptive immune responses. How are many of the potent allergens being recognized by our immune system?

As mentioned earlier the proteinase activity of the allergens may hold the answer to this question. To recap; in animal models of mucosal exposure to antigens proteinase activity of particular allergens is important for their allergenic potential (3,4). There are various theories on how the proteinase activity of allergens is involved in allergic sensitization. One proposes that allergens with proteinase activity may degrade the extra cellular matrix of the airway mucosa allowing the allergen to be more accessible to dendritic cells (DCs) (5). Another theory has these allergens cleaving cell surface molecules that are important in the regulation of the allergic response (6). Furthermore, a proteinase-mediated pathway for allergic sensitization, and subsequent development of allergic airway disease, has also been proposed (3). However, the exact mechanism by which these proteinases are involved in allergic sensitization is unknown.

Many of the potent allergens associated with atopic diseases, and more specifically asthma, are serine proteinases. Allergens with proteinase activity include HDM (7), cockroach (8), and fungal allergens whose proteinase activity is vital for the development of allergic sensitization (9). These are the same allergens that induce allergic sensitization in mice following mucosal exposure. Even potent allergens that have no proteolytic activity, such as pollen allergens, are packaged in particles that contain large quantities of proteinases (10). Finally, a number of occupational allergens also possess proteinase activity (11).

Among proteinases, serine proteinases a specific receptor system (12) through which they can affects a variety of cell types. These receptors are the PARs and encompass a family of 4 receptors (PAR-1 to PAR-4). Proteinases cleave within the

N-terminus of the receptors and unmask a tethered ligand domain that folds back on the receptor for activation. One of the members of this family, PAR-2, has been implicated in the development of inflammatory reactions (13-15), is expressed in a number of cell types in the airways (16-21) and can be activated by allergens possessing serine proteinase activity (22,23). Therefore, PAR-2 is a prime candidate to sense environmental exposures to serine proteinases. Furthermore, as shown in Chapter 2, its activation can cause an innate response in the airways consisting of the release of TNF and IL-13. As mentioned the TNF has strong immunogenic properties on DCs, while IL-13 could promote a signal to these DCs that could favor the development of Th2 lineated T cells.

We hypothesized that PAR-2 activation by an allergen or a serine proteinase inhaled concurrently with the allergen is a mechanism leading to allergic sensitization in genetically susceptible individuals. Since many aeroallergens are serine proteinases, or are presented in the airways together with serine proteinases, this hypothesis proposes a general mechanism of allergic sensitization. The susceptibility to allergic sensitization could be associated with polymorphisms in this pathway. Indeed one PAR-2 polymorphism that has been described decreases the ability of the receptor to become activated following interactions with serine proteinases (25).

Mucosal exposure of mice to HDM allergens, which are able to activate PAR-2, leads to allergic sensitization (26), while mucosal exposure to OVA leads to immune tolerance (27). To test our hypothesis we used a murine system with mucosal exposure to OVA as an Ag, and to a PAR-2AP, to mimic the potential of the allergen or other inhaled proteinases to activate PAR-2. Using this system we reproduced the PAR-2-activating potential of an allergen with serine proteinase activity, but avoided the PAR-2-independent effects of proteolytic enzymes. We further hypothesized that the mechanism by which PAR-2 activation leads to sensitization is through TNF in the airways, which influences the resulting immune response. In the present study we show that PAR-2 activation in the airways at the time of mucosal administration of OVA leads to allergic sensitization, while animals that encounter OVA alone develop tolerance. Furthermore, this allergic sensitization depends on TNF.

II. Methods

Animals

Male Balb/c and C57/B6 mice (18-20 g) were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). All mice were housed in Virus and Ab free conditions and maintained on a 12 h light-dark schedule. The University of Alberta Health Sciences Laboratory Animal Ethics Board (Edmonton, AB, Canada) approved all experiments described.

Intranasal administration of OVA and PAR-2 peptides

Following light anesthesia with ketamine (75 mg/kg) and acepromazine (6 μg/kg), groups of mice were i.n. administered 50 or 100 μg OVA (Grade V, Sigma) dissolved in 25 μl of 0.9% sterile saline 5 times once every 5th day or on 3 consecutive days respectively. For PAR-2 activation, we administered i.n. a PAR-2AP (SLIGRL-NH_2, 25 μL of 100 μM solution in saline) at the time of OVA administration. Other mice received the control peptide PAR-2CP (LSIGRL-NH_2, 25 μL of 100 μM solution in saline) or saline with OVA. All experiments were performed using 4-5 mice per group. Experiments were reproduced at least 3 times. The total number of mice studied is shown in the figure legend.

i.p. OVA immunization

Where indicated, animals were immunized with an i.p. injection of 0.9% sterile saline (0.5 mL) containing 10 μg OVA and 2 mg $Al(OH)_3$.

Airway hyperresponsiveness (AHR)

Twenty four hours after the final administration of OVA with or without PAR-2 peptides, or 24 h following aerosolized OVA challenge (5% OVA in 5 mL saline for 5 min) we measured Enhanced Pause (Penh) using whole-body plethysmography (Buxco Electronics, Inc, Sharon, CT, USA) to determine AHR to methacholine (2-32 mg/mL) as described (28). Penh was measured in mice using whole-body plethysmography to determine bronchoconstriction in response to methacholine (MeCh) challenge. Conscious mice were placed in chamber and allowed to normalize for a period of 10 min. Mice were then exposed to saline aerosol (MeCh vehicle) (3 min) and average Penh values were recorded over a 5 min period to determine baseline reactivity. Mice were then challenged using increasing concentrations of MeCh (2, 4, 8, 16, 32 µg/mL) via ultrasonic aerosol (3 min) and the average Penh values were recorded over a 5 min period following challenge. A 10 min rest period followed each 5 min Penh recording period.

In vitro T cell proliferation

Spleens from two mice from each experimental group were excised from animals after sacrifice, homogenized in HBSS, pooled, and passed through a 70 µm nylon mesh (BD PharMingen, San Diego, CA, USA) to obtain a single cell suspension. Red blood cells were lysed with Tris (20 mM) ammonium chloride (140 mM) and the single cell suspension was incubated on nylon wool columns at 37°C for 1 h to isolate T cells. Purity of T cells was ≥90% as determined by flow cytometry for CD3 (anti-CD3 antibody from BD PharMingen). T cells (2.5×10^5) were subsequently cultured in a 96 well flat bottom plate *in vitro* with 2.5×10^5 irradiated spleen cells as a source of APCs in the presence of OVA (100 µg/mL and 1000 µg/mL) in 0.2 mL DMEM containing 10% FBS, 4 mmol/L L-glutamine, and 100 µg/mL penicillin/streptomycin for 4 days. T cell proliferation was assessed using the

CellTiter 96 MTS colorimetric cell proliferation assay (Promega, Madison, WI, USA).

Cytokine assays

IL-4, IL-5, IL-10 and IL-13 were measured in the supernatants of T cell and irradiated APC co-cultures by ELISA. For the cytokine assays paired capture and biotinylated detection antibodies and recombinant protein standards were individually optimized for the assays, but otherwise employed as recommended by the supplier (R&D systems).

OVA-specific IgE

ELISA plates were coated with 50 μL of anti-IgE capture antibody (2 μg/mL) in coating buffer (0.1M $NaHCO_4$, pH 8.2) and left overnight at 4°C. Following overnight incubation plates were washed 3 times with PBS, pH 7.2, containing 0.05% Tween 20 (PBS-T) and blocked with PBS-10% FBS at room temperature for 2 h. Plates were then washed 3 times with PBS-T and either 100 μL of mouse serum samples or OVA-specific IgE standards (10000, 5000, 2500, 1250, 500, 250, 125, 75 pg) were added to the plate and left to incubate overnight at 4°C. Following incubation plates were washed 4 times with PBS-T and 100 μL of biotinylated OVA (0.5 μg/mL in PBS-10% FBS) was added to the plate and left to incubate at room temperature for 90 min. Plates were then washed 6 times with PBS-T and 100 μL of Streptavidin-HRP was added and left to incubate at room temperature for 90 min. After 90 min plates were washed 8 times with PBS-T and 100 μL of ABTS substrate was added and the reaction was allowed to develop for 15 min. Plates were then read at 405 nm on an ELISA reader.

OVA-specific IgG_1 and IgG_{2A}

Plates were coated with OVA (20 mg/mL), mouse plasma samples diluted 1:30 in PBS-T and biotinylated anti-mouse IgG_1 or IgG_{2A} (1 mg/mL) was employed as the detection reagent. IgG_1 and IgG_{2A} standard curves were generated using isotype-specific capture antibodies and recombinant immunoglobulin together with the appropriate detection antibodies.

Airway/lung inflammation

24 h following the final administrations of PAR-2AP or CP, mice were sacrificed (ketamine, 0.5g/kg; xylazine, 0.1g/kg, i.p.) trachea exposed and cannulated and lungs rinsed with PBS (7 × 0.8mL) along with chest cavity massage. 25μL of lavage fluid was diluted ½ with trypan blue and the numbers of total cells in BAL were determined using a hemocytometer. Differential cell counts of BAL were determined by staining 5×10^3 cytospun cells using Diff-Quick methodology. In other experiments lung inflammation was assessed by determining the numbers of eosinophils following lung digestion in HBSS containing 1.7 mg/mL collagenase type III (Worthington Biochemical, Lakewood, NJ) at 37°C for 1 h.

Flow cytometry (FACS®) following Alexa-488® OVA administration

Following light anesthesia with ketamine (75 mg/kg) and acepromazine (6 μg/kg), groups of mice were administered 100 μg Alexa-488® OVA i.n. (Molecular Probes Inc, Eugene, OR) suspended in 25 μL saline alone or with PAR-2AP (100 μM). Control mice received PAR-2CP (100 μM) with Alexa-488® OVA while unlabelled OVA was administered to other mice as a control. After 24 h spleen, cervical lymph node (LN), and bronchial LN were digested in 1.7 mg/mL collagenase type IV (Worthington Biochemical, Lakewood, NJ), passed through a 70 μm nylon mesh, cells from LNs pooled, and 1 × 10^6 cells stained with PE-conjugated anti-CD11c Ab. Analytical flow cytometry was carried out with a FACSCalibur® (Becton Dickinson, San Jose, CA) to determine the percentage of double positive cells. Data was processed with Cellquest software (Becton Dickinson).

TNF neutralization experiments

Mice received 100 μg goat anti-mouse TNF polyclonal Ab (R&D systems, Minneapolis, MN) or 100 μg normal goat IgG (R&D systems) 24 h before the first and 4 h before the second administration of OVA with PAR-2CP or PAR-2AP. Fifteen days later some mice were sacrificed and T cell proliferation was determined using MTS reagent while other mice were challenged 16 days following the initial administrations with i.n. OVA 4 times on alternating days and lung inflammation and AHR were assessed.

Statistical analysis

Statistical differences in the means between treatment groups were determined by using a one-way ANOVA. The paired Student *t* test was used to compare the means of 2 groups. Differences in AHR were determined by *F* test analysis that compared values over the entire curves of each treatment group to one another. From this *F* score, a *P* value was generated. In all cases, a *P* value <0.05 was considered statistically significant.

III. Results

i. PAR-2 activation promotes allergic sensitization

To model the effects of an allergen that has PAR-2 activating potential we administered OVA with a PAR-2AP. A scrambled version of this peptide was used as control (PAR-2CP). Mice were administered OVA (50 µg) alone or in combination with PAR-2AP or PAR-2CP (25 µL of 100 µM solution) i.n. once every 5th day for a total of five times (control mice received saline, PAR-2AP or PAR-2CP without OVA). Twenty-four h after the final administration mice were assessed for AHR and airway inflammation by whole body plethysmography and bronchoalveolar lavage (BAL) respectively (Fig. 3.1A). Mice receiving saline, PAR-2CP alone, PAR-2AP alone, OVA, or OVA with PAR-2CP did not develop AHR or airway inflammation. However, mice given OVA with PAR-2AP developed both AHR and airway inflammation (Fig. 3.2A and 3.2B).

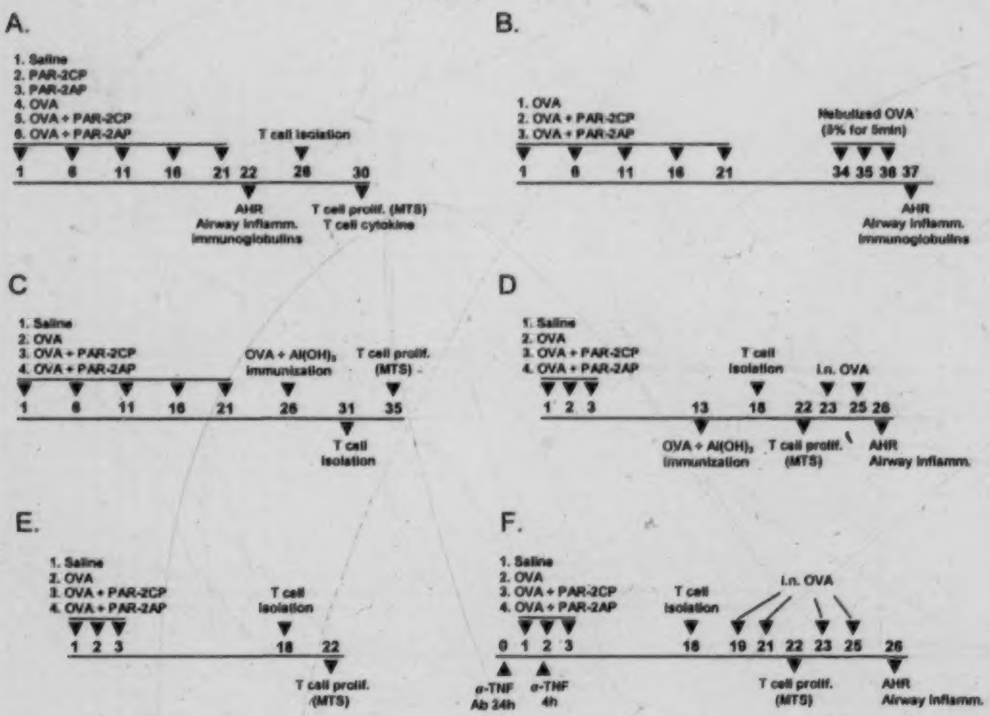

Figure 3.1. Protocols used to study the role of PAR-2 in the development of allergic sensitization.

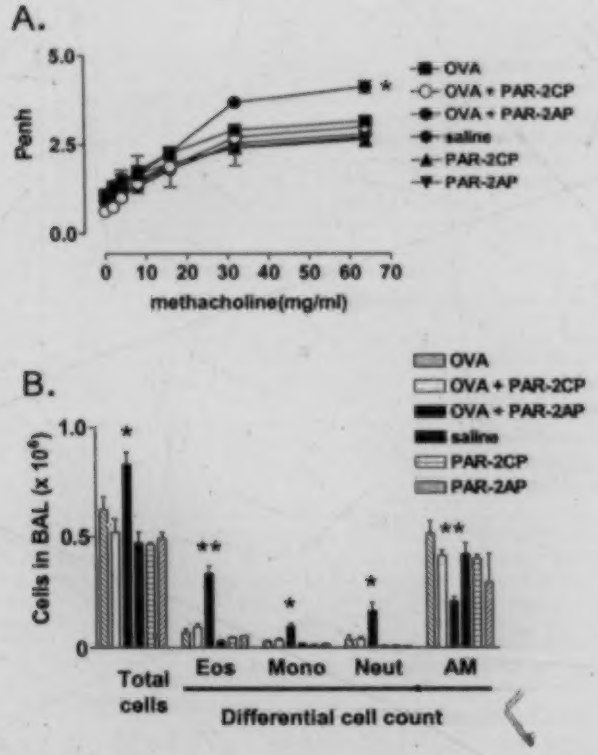

Figure 3.2. PAR-2 activation concurrently with mucosal exposure to OVA promotes AHR and airway inflammation. Mice were given 50 µg OVA alone or with PAR-2AP or PAR-2CP (25 µL of 100 µM solution) i.n. 5 times once every 5th day. Saline, PAR-2CP, or PAR-2AP were administered alone as controls. Twenty four hours after the final administration AHR and airway inflammation were assessed. (A) Enhanced pause (Penh) was measured by whole-body plethysmography to determine AHR in response to methacholine challenge (n=9). *$P < 0.05$ compared with saline, PAR-2CP administered alone, PAR-2AP administered alone, or OVA ± PAR-2CP. (B) Airway inflammation was determined by differential inflammatory cell counts in bronchoalveolar lavage (BAL) fluid (Eos; eosinophils, Mono: mononuclear, Neut: neutrophil, AM: alveolar macrophages) (n=11). *$P < 0.05$; **$P < 0.01$ compared with

saline, PAR-2CP administered alone, PAR-2AP administered alone, or OVA ± PAR-2CP. All results are shown as mean ± SEM.

Other groups of mice were sacrificed 5 days after the last i.n. administration and splenic T cells were co-cultured *in vitro* with irradiated APCs from the spleens of naïve mice in the presence of OVA. T cell proliferation and cytokine release from these T cells was assessed 4 days later (Fig. 3.1A). Splenic T cells from mice given OVA alone or OVA with PAR-2CP proliferated poorly while splenic T cells from mice given OVA with PAR-2AP proliferated vigorously (Fig. 3.3). Furthermore, T cells from mice given OVA alone or OVA with the PAR-2CP produced small amounts of IL-4, IL-5, and IL-13, and a large amount of IL-10. In contrast, splenic T cells from mice given OVA with PAR-2AP produced large amounts of IL-4, IL-5, and IL-13 and low amounts of IL-10 (Fig. 3.4), indicating the development of OVA-specific Th2 cells.

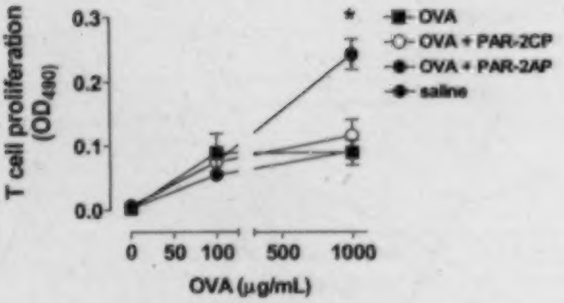

Figure 3.3. PAR-2 activation concurrently with mucosal exposure to OVA induces OVA-specific T cells that proliferate when re-stimulated with OVA in vitro. Mice were given 50 μg OVA alone or with PAR-2AP or PAR-2CP (25 μL of 100 μM solution) i.n. 5 times once every 5th day. Saline administered alone acted as a control for T cell proliferation. Five days later splenic T cells were isolated and cultured with increasing doses of OVA (100 and 1000 μg/mL) and irradiated APCs in vitro for 4 days after which proliferation was assessed. T cell proliferation was determined using MTS colorimetric cell proliferation assay read at 490 nm (n=3). $^*P < 0.05$ compared with saline, OVA administered alone, or with PAR-2CP. All results are shown as mean ± SEM.

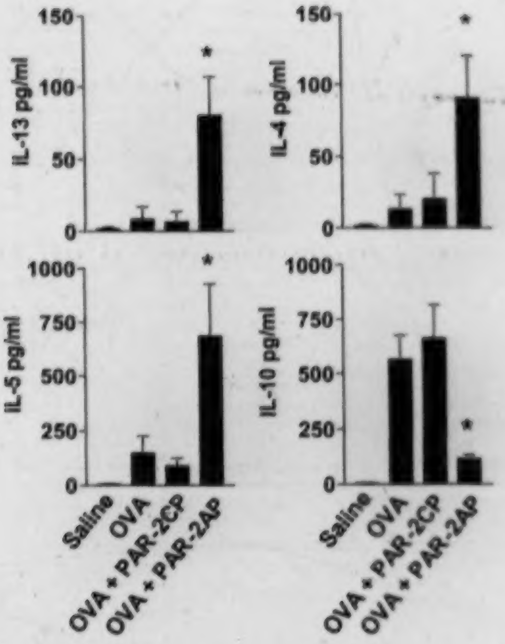

Figure 3.4. PAR-2 activation concurrently with mucosal exposure to OVA induces allergic sensitization. Mice were given 50 μg OVA alone or with PAR-2AP or PAR-2CP (25 μL of 100 μM solution) i.n. 5 times once every 5th day. Saline administered alone acted as a control for T cell proliferation. Five days later splenic T cells were isolated and cultured with increasing doses of OVA (100 and 1000 μg/mL) and irradiated APCs in vitro for 4 days after which secreted cytokines were assessed. IL-4, IL-5, IL-10, and IL-13 were measured in the supernatants of in vitro T cell proliferation cultures stimulated with 1000 μg/mL OVA in the presence of APCs by ELISA (n=3). *P < 0.05 compared with saline, OVA administered alone, or with PAR-2CP. All results are shown as mean ± SEM.

To confirm that PAR-2AP administration promoted allergic sensitization, memory responses to OVA and the production of OVA-specific Igs were assessed in mice treated as above. Two weeks after the last administration of OVA with PAR-2AP the numbers of eosinophils in the BAL returned to baseline. At this time we administered aerosolized OVA (5% OVA in 5mL saline for 5 min) on 3 consecutive days to mice from all groups (Fig. 3.1B). AHR did not differ from that seen in mice initially administered OVA alone or with PAR-2CP or PAR-2AP (Fig. 3.5A). However, mice given OVA with PAR-2AP initially showed enhanced eosinophilic airway inflammation after aerosolized OVA challenge (Fig. 3.5B). In contrast, mice given OVA with PAR-2CP initially did not exhibit signs of AHR or airway inflammation after challenge with aerosolized OVA (Fig. 3.5A and 3.5B). Furthermore, mice given OVA with PAR-2AP initially exhibited the production of OVA-specific IgE in their serum as well as slight production of OVA-specific IgG_1 and $IgG2_a$ (Fig. 3.6). The enhanced airway inflammation indicates an OVA-specific memory response in PAR-2AP treated mice. This, along with the production of OVA-specific IgE strongly indicates the possibility that PAR-2-mediated allergic sensitization.

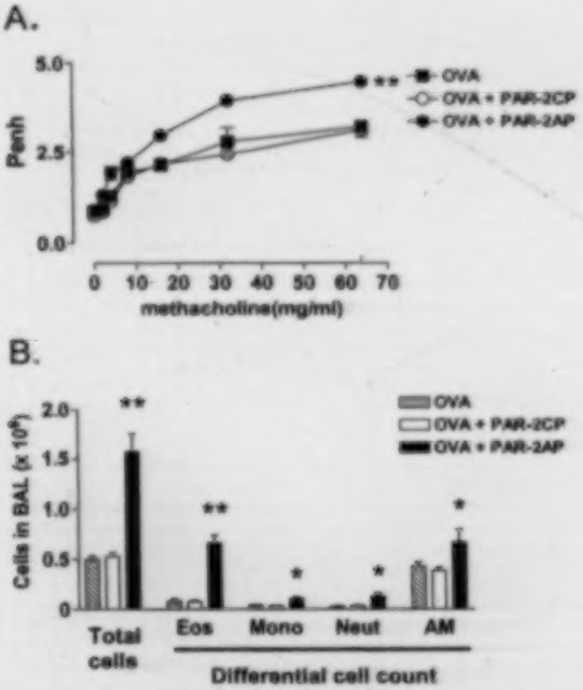

Figure 3.5. Memory response to OVA following PAR-2 activation concurrently with mucosal exposure to OVA. Mice were given 50 µg OVA alone or with PAR-2AP or PAR-2CP (25 µL of 100 µM solution) i.n. 5 times once every 5th day followed 2 wks later by aerosolized OVA challenge (5% OVA in 5 mL saline for 5 min). Twenty four hours after the final challenge AHR and airway inflammation were assessed. (A) Enhanced pause (Penh) was measured by whole-body plethysmography to determine AHR in response to methacholine challenge (n=5). *P < 0.05 compared with OVA administered alone, or with PAR-2CP. (B) Airway inflammation was determined by differential inflammatory cell counts in bronchoalveolar lavage (BAL) fluid (Eos: eosinophils, Mono: mononuclear, Neut:

neutrophil, AM: alveolar macrophages) (n=5). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with saline, OVA alone or with PAR-2CP. All results are shown as mean ± SEM.

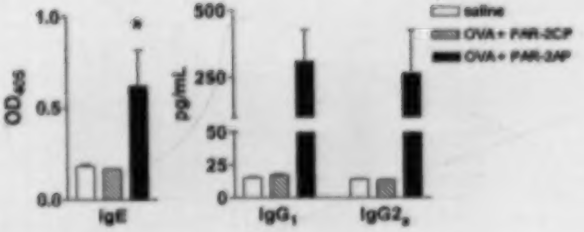

Figure 3.6. Measurement of OVA-specific Igs following PAR-2 activation concurrently with mucosal exposure to OVA. Mice were given 50 μg OVA alone or with PAR-2AP or PAR-2CP (25 μL of 100 μM solution) i.n. 5 times once every 5th day followed 2 wks later by aerosolized OVA challenge (5% OVA in 5 mL saline for 5 min). Twenty four hours after the final challenge OVA-specific IgE, IgG_1, and $IgG2_a$ in the serum of mice were determined by ELISA (n=8 OVA + PAR-2CP or AP, n=4 saline). $^{*}P < 0.05$ compared with saline or OVA with PAR-2CP. All results are shown as mean ± SEM.

The high levels of IL-10 secreted by T cells from mice given OVA alone or with PAR-2CP (Fig. 3.4) suggested the development of immune tolerance as shown previously (31). To confirm the presence of immune tolerance we immunized mice with an i.p. injection of OVA and $Al(OH)_3$ 5 days after the last i.n. administration of OVA with or without PAR-2AP (Fig. 3.1C). Splenic T cells from the mice that received OVA alone or with PAR-2CP before i.p. sensitization proliferated poorly when stimulated with OVA and APCs *in vitro*, while splenic T cells from mice given OVA with PAR-2AP proliferated vigorously (Fig. 3.7). These results confirmed the existence of immune tolerance in mice who were administered OVA alone or with the PAR-2CP, as these mice did not develop proliferating OVA-specific T cells even following i.p. immunization with OVA and $Al(OH)_3$.

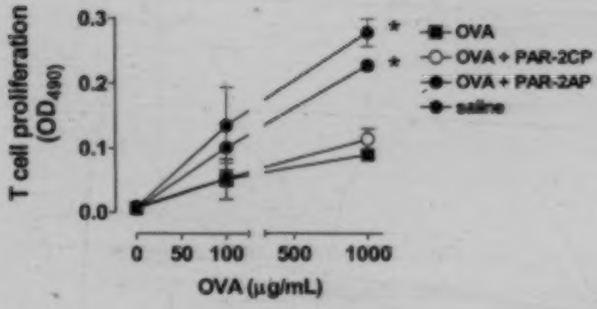

Figure 3.7. PAR-2AP administrations with OVA prevents i.n. OVA induced mucosal airway tolerance. Mice were given 50 µg OVA alone or with PAR-2CP or PAR-2AP (25 µL of 100 µM solution) i.n. 5 times once every 5th day followed by an OVA and $Al(OH)_3$ immunization as a test for OVA-specific T cell tolerance. Five days after the immunization splenic T cells were isolated from mice and cultured with increasing doses of OVA (100 and 1000 µg/mL) and irradiated APCs in vitro for 4 days, after which proliferation was assessed. T cell proliferation was determined using MTS colorimetric cell proliferation assay read at 490 nm (n=3). $*P < 0.05$ compared with OVA alone or with PAR-2CP. All results are shown as mean ± SEM.

To determine if PAR-2-mediated allergic sensitization is independent of the specific model used, we replicated these experiments in an accepted model of tolerance (32). Mice were administered 100 μg OVA i.n. alone, with PAR-2AP, or with PAR-2CP (25 μL of 100 μM) for 3 consecutive days. Control mice received saline i.n.. Ten days later all mice received an i.p. immunization with OVA and $Al(OH)_3$. Some mice were sacrificed 5 days later and splenic T cells were cultured *in vitro* with different concentrations of OVA and irradiated APCs isolated from naïve mice. T cell proliferation was assessed 4 days later (Fig. 3.1D). T cells isolated from mice, given i.n. OVA alone or with PAR-2CP initially, proliferated poorly following *in vitro* stimulation with OVA, while T cells from mice given saline or OVA with PAR-2AP proliferated vigorously (Fig. 3.8). Other groups of mice were challenged with OVA twice on alternate days starting 10 days after i.p. immunization and AHR and lung inflammation assessed 24 h after the second challenge (Fig. 3.1D). AHR was measured by plethysmography and lung inflammation was evaluated by the numbers of eosinophils in lung digests. Mice who were administered OVA alone or OVA with PAR-2CP initially did not develop AHR or lung inflammation following OVA challenge, while mice given saline or OVA with PAR-2AP did (Fig. 3.9A and 3.9B). These experiments showed that mice receiving OVA with PAR-2AP did not develop tolerance, in contrast to mice receiving OVA alone or with PAR-2CP.

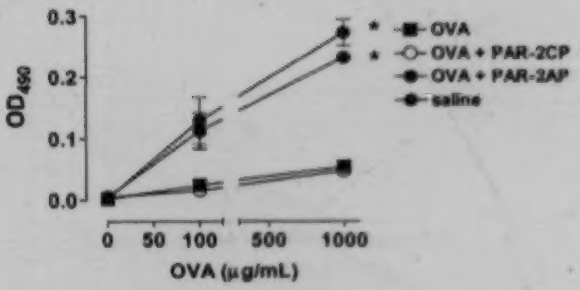

Figure 3.8. PAR-2 activation prevents mucosal airway tolerance to OVA. Mice were given saline, 100 µg OVA alone or with PAR-2AP or PAR-2CP (25 µL of 100 µM solution) i.n. on 3 consecutive days followed by i.p. OVA and $Al(OH)_3$ immunization. Five days after the immunization splenic T cells were isolated from mice and cultured with increasing doses of OVA (100 and 1000 µg/mL) and irradiated APCs in vitro for 4 days, after which proliferation was assessed. T cell proliferation was determined using MTS colorimetric cell proliferation assay read at 490 nm (n=3). *$P < 0.05$ compared with OVA alone or with PAR-2CP. All results are shown as mean ± SEM.

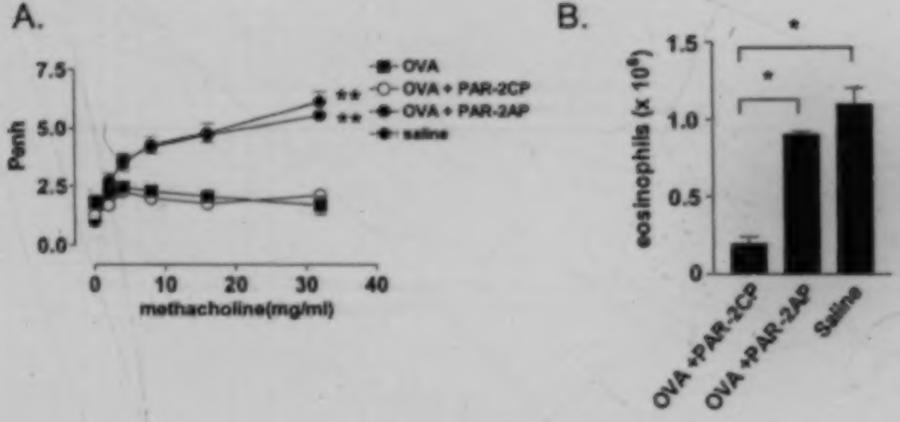

Figure 3.9. PAR-2 activation prevents mucosal airway tolerance to OVA. Mice were given saline, 100 μg OVA alone or with PAR-2AP or PAR-2CP (25 μL of 100 μM solution) i.n. on 3 consecutive days followed by i.p. OVA and $Al(OH)_3$ immunization. Ten days after the immunization mice were challenged twice i.n. with 50 μg OVA and assessed for AHR and airway inflammation 24 h after the 2nd challenge. (A) Enhanced pause (Penh) was measured by whole-body plethysmography to determine AHR in response to methacholine challenge (n=9). **$P < 0.01$ compared with OVA administered alone, or with PAR-2CP. (B) Airway inflammation was determined by the number of eosinophils isolated following lung digestion (n=4). *$P < 0.05$ compared with OVA administered with PAR-2CP.

To study whether OVA with PAR-2AP also induced allergic sensitization in this model we repeated the protocol of i.n. sensitization on 3 consecutive days, without subsequent i.p. immunization, and assessed allergic sensitization by T cell proliferation to OVA (Fig. 3.1E). T cells isolated from mice that were administered OVA with PAR-2AP i.n. proliferated vigorously indicating that these animals were sensitized to the OVA. T cells from mice given OVA alone or with PAR-2CP proliferated poorly (Fig. 3.10).

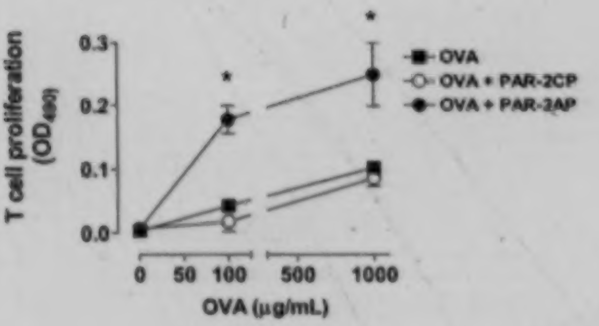

Figure 3.10. PAR-2 activation concurrently with mucosal exposure to OVA induces sensitization. Mice were given 100 µg OVA alone or with PAR-2AP or PAR-2CP (25 µL of 100 µM solution) i.n. on 3 consecutive days and 15 days later splenic T cells were isolated and cultured with increasing doses of OVA (100 and 1000 µg/mL) and irradiated APCs in vitro for 4 days, after which proliferation was assessed. T cell proliferation was determined using MTS colorimetric cell proliferation assay (n=3). $^*P < 0.05$ compared with OVA administered alone, or with PAR-2CP. All results are shown as mean ± SEM.

To study whether the PAR-2 induced effects on sensitization were mouse strain specific we addressed whether OVA with PAR-2AP induced allergic sensitization in the C57/B6 strain of mouse. We repeated the protocol of i.n. sensitization on 3 consecutive days, without subsequent i.p. immunization, and assessed allergic sensitization by T cell proliferation to OVA (Fig. 3.1E). As a positive control other mice were given a single i.p. immunization of OVA + $Al(OH)_3$ and T cells from these mice were isolated 5 days following immunization. T cells isolated from mice that were administered OVA with PAR-2AP i.n. or given OVA + $Al(OH)_3$ immunization proliferated vigorously indicating that these animals were sensitized to the OVA. T cells from mice given OVA with PAR-2CP proliferated poorly (Fig. 3.11).

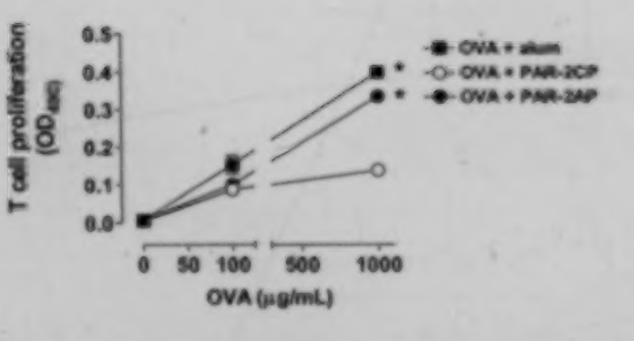

Figure 3.11. PAR-2 activation with mucosal exposure to OVA induces sensitization in C57/B6 mice. C57/B6 mice were given 100 µg OVA with PAR-2AP or PAR-2CP (25 µL of 100 µM solution) i.n. on 3 consecutive days and 15 days later splenic T cells were isolated and cultured with increasing doses of OVA (100 and 1000 µg/mL) and irradiated APCs in vitro for 4 days, after which proliferation was assessed. As a positive control other mice were given a single i.p. immunization of OVA + $Al(OH)_3$ and T cells from these mice were isolated concurrently with the mice given OVA with PAR-2 peptides 5 days following immunization. T cell proliferation was determined using MTS colorimetric cell proliferation assay (n=3). $^*P < 0.05$ compared with OVA administered with PAR-2CP. All results are shown as mean ± SEM.

ii. PAR-2 activation in the airways alters pulmonary DC trafficking

Inhaled Ag is taken up and processed by CD11c-positive DCs and presented to naïve T cells in the draining LNs and the spleen. To investigate the effects of PAR-2 activation in the airways on DC activation status, we studied Ag uptake by DC and their accumulation in the cervical and bronchial LNs and the spleen using Alexa-488®-labeled OVA (FL-OVA). Unlabelled OVA acted as a control. FL-OVA was administered alone or in combination with PAR-2AP or PAR-2CP (25 µL of 100 µM) and either 6, 12, 24, 48, or 72 h later spleens, and pooled cervical and bronchial LNs, were digested and the cell suspension stained for CD11c as a DC marker and analyzed by flow cytometry. PAR-2 activation in the airways enhanced the uptake of FL-OVA by CD11c$^+$ DCs in the spleen when compared to FL-OVA given alone or with PAR-2CP 6 h after administration (Fig. 3.12). This enhancement to FL-OVA uptake rose at the 12 h assessment (Fig. 3.13) and reached a maximum at 24 h (Fig. 3.14). After 24 h the enhancement began to diminish as smaller differences were seen at the 48 h (Fig. 3.15) and 72 h (Fig. 3.16) assessments. Therefore, PAR-2 activation in the lungs augments antigen uptake by DCs and increases DC numbers residing in the spleen reaching a peak 24 h after Ag uptake.

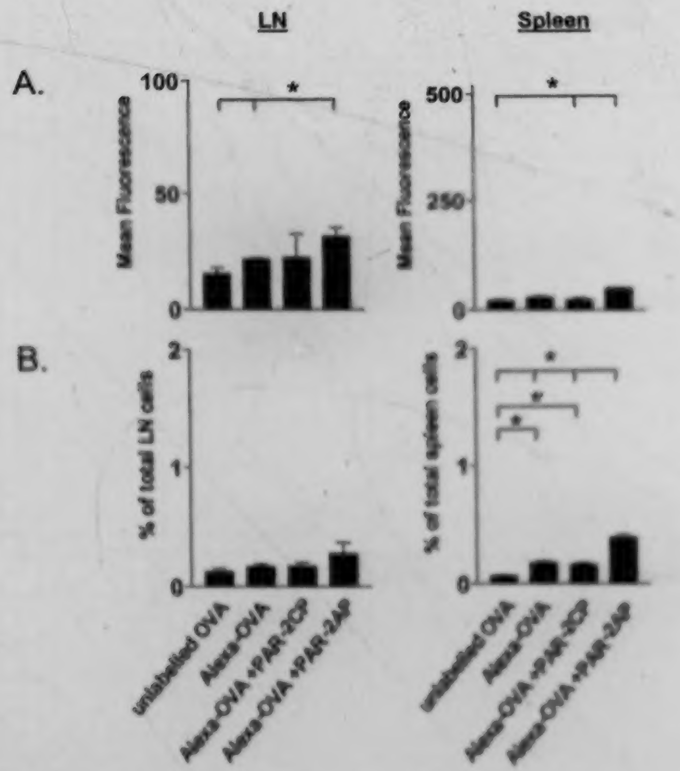

Figure 3.12. DC antigen uptake and trafficking 6 h following PAR-2 activation. (A) Mean fluorescent intensity of Alexa-488® OVA (FL-OVA) in double positive CD11c$^+$ FL-OVA$^+$ dendritic cells from the cervical and bronchial LNs and spleen 6 h following administration of 100 µg FL-OVA alone with PAR-2AP, or PAR-2CP (25 µL of 100 µM solution). Unlabelled OVA acted as a control (n=2). *P < 0.05 compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM. (B) Percentage of CD11c$^+$, FL-OVA$^+$ double positive cells in the LNs and spleen 6 h following administration of 100 µg FL-OVA alone with PAR-2AP, or PAR-2CP (n=2). *P < 0.05 compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM.

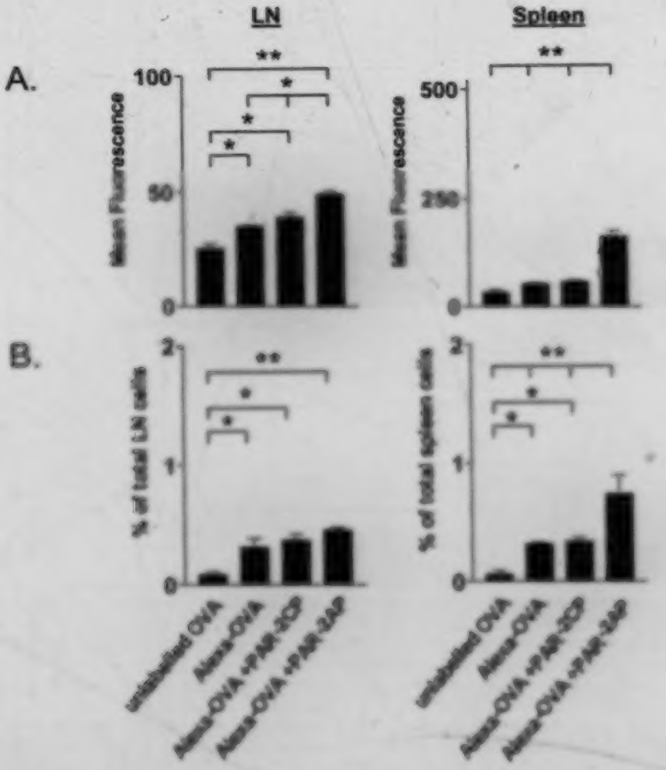

Figure 3.13. DC antigen uptake and trafficking 12 h following PAR-2 activation. (A) Mean fluorescent intensity of Alexa-488® OVA (FL-OVA) in double positive CD11c$^+$ FL-OVA$^+$ dendritic cells from the cervical and bronchial LNs and spleen 12 h following administration of 100 μg FL-OVA alone with PAR-2AP, or PAR-2CP (25 μL of 100 μM solution). Unlabelled OVA acted as a control (n=2). $^*P < 0.05$; $^{**}P < 0.01$ compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM. (B) Percentage of CD11c$^+$, FL-OVA$^+$ double positive cells in the LNs and spleen 12 h following administration of 100 μg FL-OVA alone with PAR-2AP, or PAR-2CP (n=2). $^*P < 0.05$; $^{**}P < 0.01$ compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM.

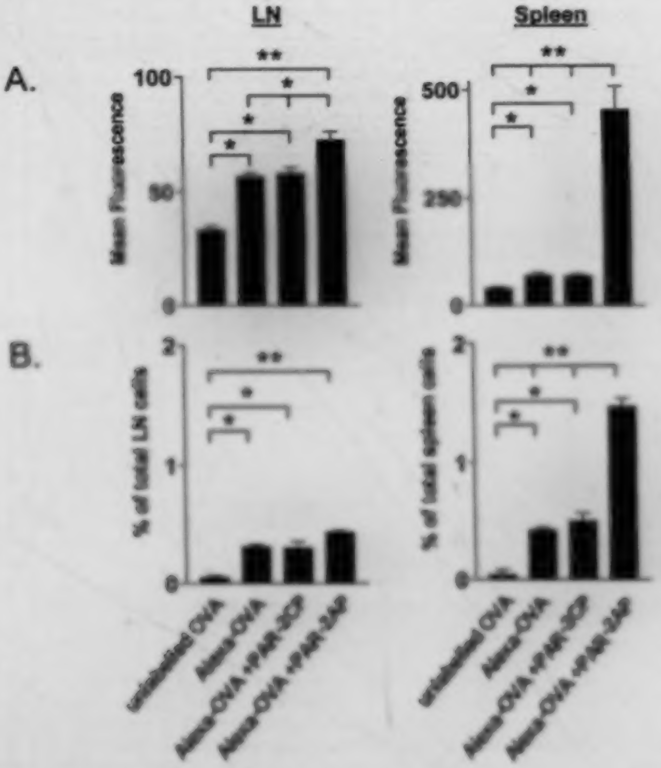

Figure 3.14. DC antigen uptake and trafficking 24 h following PAR-2 activation. (A) Mean fluorescent intensity of Alexa-488® OVA (FL-OVA) in double positive CD11c$^+$ FL-OVA$^+$ dendritic cells from the cervical and bronchial LNs and spleen 24 h following administration of 100 µg FL-OVA alone with PAR-2AP, or PAR-2CP (25 µL of 100 µM solution). Unlabelled OVA acted as a control (n=3). $^*P < 0.05$; $^{**}P < 0.01$ compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM. (B) Percentage of CD11c$^+$, FL-OVA$^+$ double positive cells in the LNs and spleen 24 h following administration of 100 µg FL-OVA alone with PAR-2AP, or PAR-2CP (n=3). $^*P < 0.05$; $^{**}P < 0.01$ compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM.

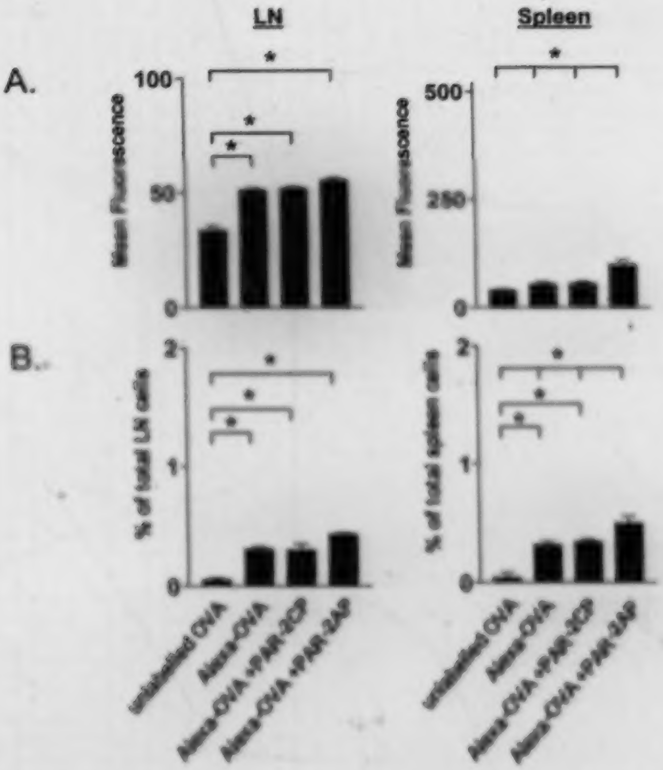

Figure 3.15. DC antigen uptake and trafficking 48 h following PAR-2 activation. (A) Mean fluorescent intensity of Alexa-488® OVA (FL-OVA) in double positive CD11c$^+$ FL-OVA$^+$ dendritic cells from the cervical and bronchial LNs and spleen 48 h following administration of 100 μg FL-OVA alone with PAR-2AP, or PAR-2CP (25 μL of 100 μM solution). Unlabelled OVA acted as a control (n=2). *P < 0.05 compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM. (B) Percentage of CD11c$^+$, FL-OVA$^+$ double positive cells in the LNs and spleen 48 h following administration of 100 μg FL-OVA alone with PAR-2AP, or PAR-2CP (n=2). *P < 0.05 compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM.

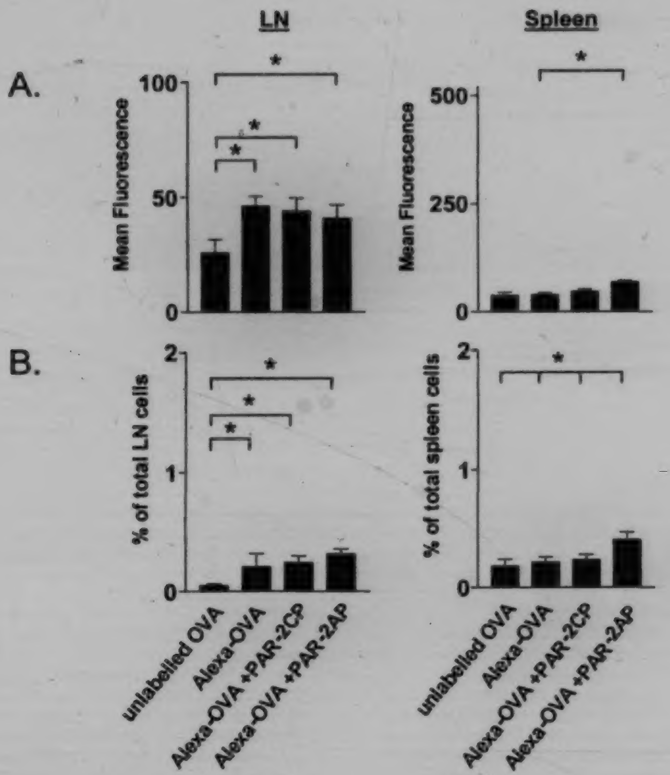

Figure 3.16. DC antigen uptake and trafficking 72 h following PAR-2 activation. (A) Mean fluorescent intensity of Alexa-488® OVA (FL-OVA) in double positive CD11c$^+$ FL-OVA$^+$ dendritic cells from the cervical and bronchial LNs and spleen 72 h following administration of 100 µg FL-OVA alone with PAR-2AP, or PAR-2CP (25 µL of 100 µM solution). Unlabelled OVA acted as a control (n=2). *$P < 0.05$ compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM. (B) Percentage of CD11c$^+$, FL-OVA$^+$ double positive cells in the LNs and spleen 72 h following administration of 100 µg FL-OVA alone with PAR-2AP, or PAR-2CP (n=2). *$P < 0.05$ compared with FL-OVA administered alone, with PAR-2CP, or unlabelled OVA. All results are shown as mean ± SEM.

iii. PAR-2 activation promotes allergic sensitization through the release of TNF

The above results indicate that PAR-2 activation in the airways alters DC activation status. We have previously shown that PAR-2 activation induces TNF release in the airways (24). Since TNF can activate DCs (33-35) we hypothesized that TNF released, as a result of PAR-2 activation, may alter pulmonary DC activation resulting in allergic sensitization instead of tolerance. To study the role of TNF in PAR-2-mediated allergic sensitization, we administered a TNF neutralizing antibody to mice that were also given OVA with the PAR-2AP (Fig. 3.1F). Allergic sensitization in these mice was assessed by splenic T cell proliferation. Additionally, sensitization of other mice was assessed by the number of eosinophils recruited into the lungs and the presence of AHR following challenge with OVA 16 days post sensitization. The TNF neutralizing Ab had no effect on T cell proliferation in mice receiving OVA alone or with PAR-2CP. However, TNF neutralization prevented T cell proliferation in mice given PAR-2AP with OVA (Fig. 3.17), indicating that the TNF neutralizing Ab prevented allergic sensitization. As expected, from the proliferation results we have shown in Fig. 9, mice initially given OVA and PAR-2AP with the control antibody developed airway inflammation and AHR after challenge. Conversely, mice given OVA and PAR-2AP with anti-TNF neutralizing Ab showed neither eosinophil recruitment into the lungs nor AHR, indicating the absence of sensitization (Fig. 3.18A and 3.18B). These mice had numbers of eosinophils and AHR that were similar to mice who developed tolerance. Thus, we concluded that TNF released as a result of PAR-2 activation in the airways mediates allergic sensitization to concurrently inhaled Ag.

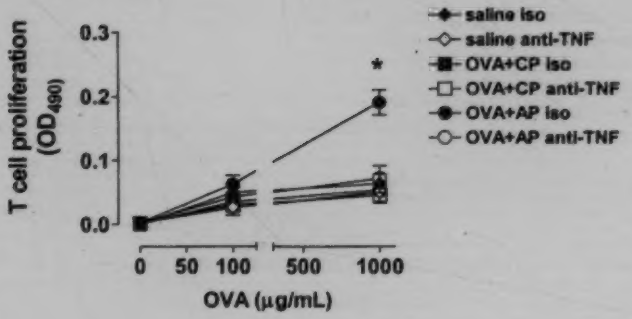

Figure 3.17. PAR-2 induced allergic sensitization is TNF dependent. Mice were administered neutralizing TNF Ab or isotype control Ab i.p. 24 h before the first and 4 h before the second of three i.n. administrations of OVA with PAR-2AP or PAR-2CP. Fifteen days later splenic T cells were isolated and cultured with increasing doses of OVA (100 and 1000 µg/mL) and irradiated APCs in vitro for 4 days, after which proliferation was assessed. T cell proliferation was determined using MTS colorimetric cell proliferation assay (n=3). $^*P < 0.05$ compared with other treatments groups. All results are shown as mean ± SEM.

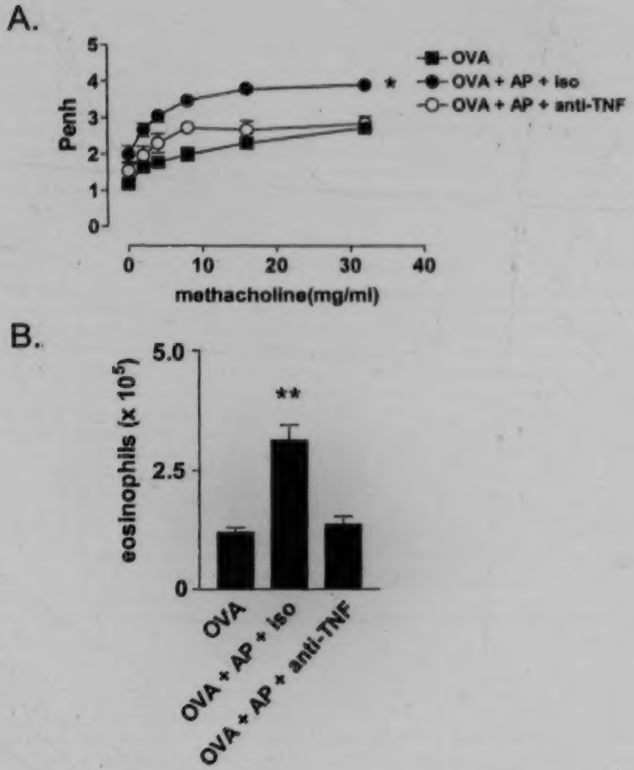

Figure 3.18. PAR-2 induced allergic sensitization is TNF dependent. Mice were challenged 4 times, starting 16 days after the initial administrations, on alternating days i.n. with 50 μg OVA and assessed for AHR 24 h after the 4th challenge. (B) Enhanced pause (Penh) was measured by whole-body plethysmography to determine AHR in response to methacholine challenge (n=5). $^*P < 0.05$ compared with OVA administered alone or with PAR-2AP and neutralizing Ab. (C) Airway inflammation was determined by the number of eosinophils isolated following lung digestion (n=5). $^{**}P < 0.01$ compared with OVA administered alone or with PAR-2AP and neutralizing Ab. All results are shown as mean ± SEM.

IV. Discussion

Although much is known about the immune mechanisms leading to allergic sensitization (36), little is known about the specific characteristics of inhaled Ags that mediate or facilitate allergic sensitization. Our results point towards a pathway in which Ags with serine proteinase activity activate PAR-2 in the airways and induce TNF release, which in turn, biases the immunological outcome towards allergic sensitization. This study, therefore, implicates PAR-2 activation as a vital step in allergic sensitization to the major allergens associated with asthma.

Previous reports have shown evidence of a proteinase-mediated pathway for allergic sensitization. Fungal extracts with proteinase activity prevented inhalation-induced tolerance to OVA and led to allergic sensitization to OVA and subsequent development of allergic airway disease (3). In a similar fashion, HDM extracts can facilitate allergic sensitization to simultaneously administered OVA (26). However, these studies did not define the mechanism of proteinase-induced allergic sensitization. For the first time here we have shown a specific receptor that may mediate these effects, namely PAR-2, and also that PAR-2-mediated allergic sensitization is dependent on TNF release.

Antigens with serine proteinase activity, such as HDM, have been shown to induce allergic sensitization of mice following mucosal exposure (37). Our study indicates that mucosal sensitization to HDM may be the result of PAR-2 activation in the airways. Studies to test this hypothesis will improve our understanding of allergic sensitization, but they are not easy to perform. The use of PAR-2 knock-out (KO) mice could show lack of mucosal sensitization to HDM in the absence of PAR-2, but there are potential problems with this approach. PAR-2 KO mice develop significantly lower AHR and airway inflammation compared to normal controls (38), and these mice may have DC developmental defects and therefore, Ag presentation may be greatly hindered *in vivo* (39). Thus, one would be unable to discern whether an apparent lack of allergic sensitization is due to the absence of PAR-2 in the airways or defects in Ag presentation.

We used a synthetic peptide to activate PAR-2 in the airways. This PAR-2AP is specific for PAR-2, as has been determined *in vivo* through the use of PAR-2 KO mice (40-42). We activated PAR-2 with 2.5 nmoles of PAR-2AP. This amount is similar to that used in other *in vivo* studies (43), including a recent one by our laboratory showing that PAR-2 activation during allergen challenge enhances AHR and airway inflammation (24). Along with this activating peptide we administered OVA, an Ag that is unable to activate PAR-2 and promotes the development of tolerance when administered in the airways. We used such a system to reconstitute the effects of proteolytic allergens that are able to activate PAR-2 and avoid other proteinase effects of the allergens on the airways.

In chapter 1 we showed that PAR-2 is expressed on epithelial cells, smooth muscle, and alveolar macrophages (AM) in the airways of Balb/c mice (24). Also mentioned in section IV of chapter 1 AM are an attractive target for further study because they are situated throughout the airways, are one of the first cell types to encounter inhaled serine proteases and can produce large amounts of TNF (44). TNF may have direct effects on DCs in the airways (45), or it may induce the release of other inflammatory mediators from the epithelium (46), which could then mediate DC activation. One such mediator, GM-CSF, has been shown to activate DCs (47) and induce allergic sensitization to OVA when administered directly or as transgene expression in the airways (48).

However other cell types, such as mast cells, have been shown to express PAR-2 in other tissues (49). Therefore, another source of TNF following PAR-2 activation could be mast cells situated throughout the airways (50). More interestingly, it has been shown that TNF derived from mast cells can enhance dendritic cell migration (45) and therefore affect the subsequent immune response. Therefore, TNF may be secreted following PAR-2 activation of one or more of the cell types discussed here. Alternatively it could be released by other cells activated by pro-inflammatory products induced by PAR-2 activation.

Our results indicate that PAR-2 activation in the airways by proteolytic allergens modifies local immune responses. PAR-2 may serve a biological purpose similar to that of TLRs (51). By recognizing conserved protein "patterns" of varying degrees of heterogeneity, PAR-2 senses "danger signals" in the environment, albeit serine proteinases instead of bacterial or viral components, and its activation influences the development of both innate immune responses, namely inflammation, and adaptive immune responses, namely the decision of the immune system to respond to a foreign antigen with immunologic tolerance or allergic sensitization. Furthermore, it has been shown that LPS activating TLR-4 in the airway is also capable of promoting allergic sensitization to inhaled proteins (52). Therefore, the activation of both PAR-2 and TLRs are capable of linking innate immunity with allergic immune responses. For this reason we were careful to ensure that our PAR-

2AP and OVA preparations had undetectable LPS levels, to exclude possible TLR involvement in our results.

The majority of protein antigens encountered in the airways induce antigen-specific immune tolerance. We have shown that PAR-2 activation in the airways at the time of Ag encounter is capable of shifting the resulting immune outcome towards allergic sensitization and development of asthma. PAR-2 antagonists, or neutralization of inflammatory mediators released in the airways following PAR-2 activation, may therefore be a very attractive therapeutic strategy to block sensitization towards major asthma-causing allergens. By blocking allergic sensitization, a tolerogenic pathway should be induced to these proteolytic allergens, which would be protective during subsequent environmental exposure to the same allergen. This would prevent the development of allergic disease.

V. References

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Chapter 4: Results section #3

The role of PAR-2 activation during the effector phase of allergic lung disease

Adapted from:

Ebeling, C., P. Forsythe, J. Ng, J. R. Gordon, M. Hollenberg, and H. Vliagoftis. 2005. Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. *J Allergy Clin Immunol* 115:623-630.

All experiments were designed and analysized by Ebeling, C and H. Vliagoftis

P. Forsythe – helped Ebeling, C with developing murine airway inflammation models

J. Ng – assisted Ebeling, C in initial experiments

J. R. Gordon – measured OVA-specific IgE

M. Hollenberg – provided the PAR-2AP and CPs

Lakshmi Puttagunta – helped with the analysis of lung sections in mice chgallenged with OVA with PAR-2CP or PAR-2AP

I. Introduction

In chapter 3 we showed that PAR-2 activation was able to promote allergic sensitization through a TNF mediated pathway. As mentioned in chapter 1 there is mounting evidence that proteinases are involved in the pathogenesis of asthma. Mast cell tryptase, a serine proteinase released by mast cells, is increased in bronchoalveolar lavage (BAL) fluid of asthmatics following allergen challenge (1) and can induce smooth muscle hyper-reactivity (2,3). Furthermore, many aeroallergens associated with asthma such as house dust mite allergens and various fungal allergens, are proteinases (4-6).

PAR-2 is of particular interest in asthma because it can be activated by mast cell tryptase (7,8), and aeroallergens (9,10) and has been implicated in inflammation. PAR-2 activation induces the release of GM-CSF, eotaxin (11), and MMP-9 (12) from airway epithelial cells. These mediators could be involved in eosinophil recruitment into the lung. PAR-2 agonist injected into rat hindpaws induces oedema and granulocyte infiltration (13). PAR-2 mediates microvascular inflammation by inducing P-selectin mediated leukocyte rolling (14), and participates in the induction of intestinal inflammation (15).

Little is known about the pathophysiological importance of PAR-2 in the airways. PAR-2 is up regulated on the airway epithelium of asthmatics (16). Transgenic mice over expressing PAR-2 develop increased levels of OVA-mediated eosinophilic airway inflammation and AHR compared to wild-type mice (17), while PAR-2 knockout mice show the opposite effect. Others have shown a possible protective role for PAR-2 in the airways. PAR-2 activation relaxes isolated rat tracheas (18) and has a protective effect against bronchoconstriction *in vivo* (19). It is therefore not clear whether PAR-2 activated in the airway is protective, i.e. induces bronchodilation, or detrimental by inducing inflammation, bronchoconstriction and/or hyperresponsiveness.

We hypothesized that PAR-2 activation in the airways has pro-inflammatory effects. Here we report that PAR-2 activation in the respiratory tract increases allergen-mediated accumulation of eosinophils and mononuclear cells in the airways and increases AHR and does so by different mechanisms.

II. Methods

Animals

Male Balb/c mice (18-20 g) were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). All mice were housed in Virus and Ab free conditions and maintained on a 12 h light-dark schedule. The University of Alberta Health Sciences Laboratory Animal Ethics Board (Edmonton, AB, Canada) approved all experiments described.

Sensitization and challenge with ovalbumin (OVA)

Male Balb/c mice were sensitized on days 1 and 6 via intraperitoneal (i.p.) injection of 0.9% sterile saline (0.5 ml) containing 10 μg OVA and 2 mg $Al(OH)_3$. Following light anesthesia with ketamine (75 mg/kg) and acepromazine (6 μg/kg) groups of mice were challenged intranasally with 25 μl of PAR-2ap (SLIGRL-NH_2, 100 μM) or PAR-2cp (LSIGRL-NH_2, 100μM) with OVA (50 μg) on days 12 and 14. Concentrations of peptides chosen were similar to those used in other *in vivo* animal studies (19,20). Mice were then assessed for airway inflammation and AHR on days 15 or 19 (short-term and follow up protocols respectively, Fig. 4.1A and 4.1B). Other mice were further challenged with saline, PAR-2ap or PAR-2cp on days 16 and 18 and analyzed on day 19 (long term protocol, Fig. 4.1C).

Bronchoalveolar lavage (BAL)

On day 15, 24 h following the final administrations of PAR-2AP or CP, mice were sacrificed (ketamine, 0.5g/kg; xylazine, 0.1g/kg, i.p.) trachea exposed and cannulated and lungs rinsed with PBS (7 × 0.8mL) along with chest cavity massage. 25μL of lavage fluid was diluted ½ with trypan blue and the numbers of total cells in

BAL were determined using a hemocytometer. Differential cell counts of BAL were determined by staining 5×10^3 cytospun cells using Diff-Quick methodology.

Airway hyperresponsiveness (AHR)

On day 15, 24 h following the final administrations of PAR-2AP or CP, enhanced pause (Penh) was measured in mice using whole-body plethysmography to determine bronchoconstriction in response to methacholine (MeCh) challenge. Conscious mice were placed in chamber and allowed to normalize for a period of 10 min. Mice were then exposed to saline aerosol (MeCh vehicle) (3 min) and average Penh values were recorded over a 5 min period to determine baseline reactivity. Mice were then challenged using increasing concentrations of MeCh (2, 4, 8, 16, 32 µg/mL) via ultrasonic aerosol (3 min) and the average Penh values were recorded over a 5 min period following challenge. A 10 min rest period followed each 5 min Penh recording period.

PAR-2 localization in BAL cells by Immunohistochemistry

Cytospins of BAL cells on slides were prepared and placed in cold acetone (20°C) for 10min, washed in PBS (5 min), placed in methanol containing 0.3% H_2O_2 for 35 min to block endogenous peroxidases then incubated with the anti-PAR-2 B5 Ab [23] (1/100 dil in 100 µl PBS) or anti-PAR-2 B5 Ab preabsorbed with immunizing peptide (65 µg/ml in 100 µl of PBS for 1h at 4°C) for 2.5h at room temp. Sections were then washed in PBS (5min), incubated with HRP labeled $F(ab')_2$ goat anti-rabbit IgG (1/1000 dil in 100 µl PBS; Jackson ImmunoResearch Inc., West Grove, PA, USA) for 1.5h at room temp, washed in PBS (5min) then stained with diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA, USA).

Sections were then rinsed in water (5 min), counterstained with Harris' Hematoxylin, and dehydrated in increasing ethanol/xylene gradient. Slides were photographed using a Nikon DXM 1200 digital camera mounted on a Nikon E 800 microscope (Nikon Corporation, Tokyo, Japan).

BAL Cytokine Assays

IL-4, IL-5, IL-9, IL-10, IL-13, TNF, and IFN-γ was measured in BAL samples by ELISA. For the cytokine assays paired capture and biotinylated detection antibodies and recombinant protein standards were individually optimized for the assays, but otherwise employed as recommended by the supplier (R&D systems).

OVA-specific IgE

ELISA plates were coated with 50μL of capture IgE (2μg/mL) in coating buffer (0.1M $NaHCO_4$, pH 8.2) and left overnight at 4°C. Following overnight incubation plates were washed 3 times with PBS, pH 7.2, containing 0.05% Tween 20 (PBS-T) and blocked with PBS-10% FBS at room temperature for 2 hours. Plates were then washed 3 times with PBS-T and either 100 μL of mouse serum samples or OVA-specific IgE standards (10000, 5000, 2500, 1250, 500, 250, 125, 75 pg) were added to the plate and left to incubate overnight at 4°C. Following incubation plates were washed 4 times with PBS-T and 100 μL of biotinylated OVA (0.5μg/mL in PBS-10% FBS) was added to the plate and left to incubate at room temperature for 90 min. Plates were then washed 6 times with PBS-T and 100 μL of Streptavitin-HRP was added and left to incubate at room temperature for 90 min. After 90 min plates were washed 8 times with PBS-T and 100 μL of ABTS substrate was added and the reaction was allowed to develop for 15 min. Plates were then read at 405 nm on an ELISA reader.

Statistical analysis

Statistical differences in the means of inflammatory cell counts between treatment groups were determined using one-way ANOVA. The paired Student *t* test was used to compare the means of 2 groups. Differences in AHR were determined by *F* test analysis that compared the entire curves of each treatment group with one another. From this *F* test a *P* value was generated. In all cases, a *P* value of less than .05 was considered statistically significant.

III. Results

i. Effects of PAR-2 activation on allergen mediated airway inflammation and AHR

We studied the effects of PAR-2 activation on OVA-induced airway inflammation and AHR. OVA sensitized mice were challenged twice with OVA alone or in combination with PAR-2AP or PAR-2CP on days 12 and 14. Inflammation and AHR were assessed the day after the last challenge (short-term protocol Fig. 4.1A). Co-administration of PAR-2AP with OVA increased total inflammatory cell numbers in the BAL ($2.2 \pm 0.4\times10^6$) compared to OVA administered alone ($1.3 \pm 0.1\times10^6$) or with PAR-2CP ($1.0 \pm 0.1\times10^6$) (Fig. 4.2A upper panel). OVA + PAR-2AP treated mice had increased numbers of eosinophils ($1.8 \pm 0.3\times10^6$) and mononuclear cells ($2.8 \pm 0.5\times10^5$) when compared to OVA given alone ($1.0 \pm 0.3\times10^6$; $p<0.05$ and $1.0 \pm 0.4\times10^5$; $p<0.01$ respectively) or with PAR-2CP ($0.9 \pm 0.1\times10^6$; $p<0.05$ and $0.8 \pm 0.2\times10^5$; $p<0.01$ respectively). OVA plus PAR-2AP treated mice exhibited a significantly higher MeCh responsiveness when compared to OVA given alone or with the PAR-2CP ($p<0.01$) (Fig 4.2A lower panel). Thus, PAR-2 activation following i.n. administration of PAR-2AP enhanced OVA-mediated allergic airway inflammation and AHR.

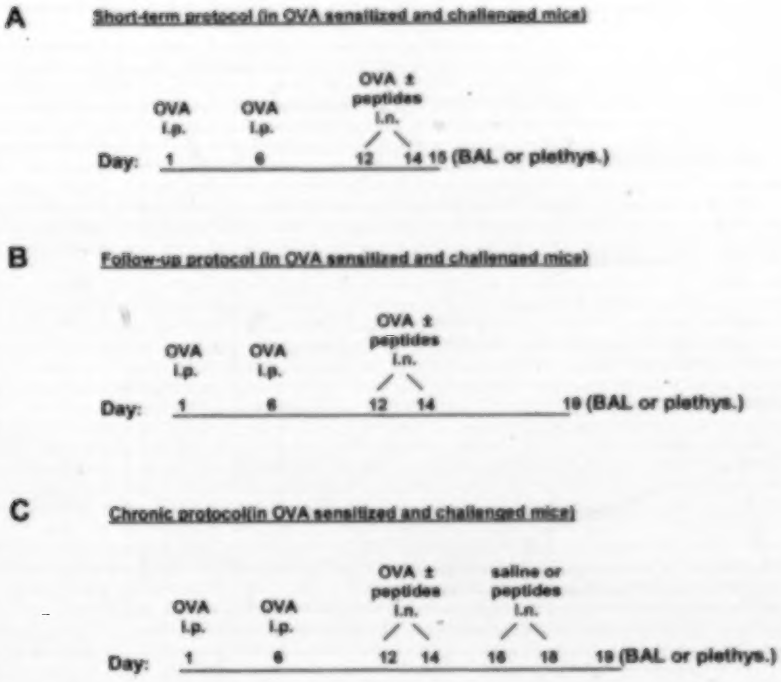
A
Short-term protocol (in OVA sensitized and challenged mice)
OVA i.p.
OVA i.p.
OVA ± peptides i.n.
Day: 1 6 12 14 15 (BAL or plethys.)
B
Follow-up protocol (in OVA sensitized and challenged mice)
OVA i.p.
OVA i.p.
OVA ± peptides i.n.
Day: 1 6 12 14 19 (BAL or plethys.)
C
Chronic protocol(in OVA sensitized and challenged mice)
OVA i.p.
OVA i.p.
OVA ± peptides i.n.
saline or peptides i.n.
Day: 1 6 12 14 16 18 19 (BAL or plethys.)

Figure 4.1. OVA sensitization/challenge protocols used to study the role of PAR-2 on airway inflammation and AHR. i.n., Intranasal; i.p., intraperitoneal; OVA, ovalbumin; plethys, plethysmography.

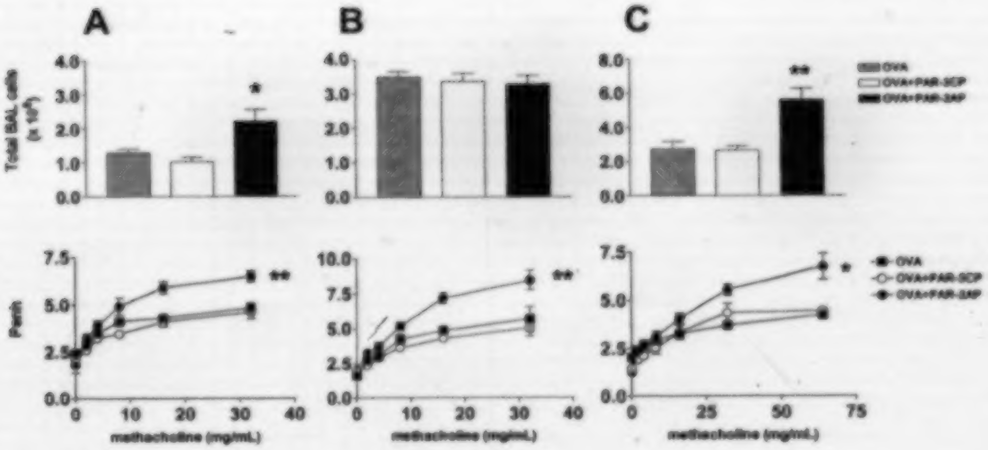

Figure 4.2. BAL total cell numbers (upper panels) and enhanced pause values from increasing doses of methacholine (lower panels) in ovalbumin-sensitized mice subject to the (A) short-term (n = 11) (B), follow-up (n = 9), and (C) chronic (n = 9) activation protocols as outlined in Fig 1. Hatched columns and squares represent OVA given alone, open columns/circles represent OVA + PAR-2CP treatment, whereas closed columns/circles represent OVA + PAR-2AP treatment. Results are shown as means ± SEMs. *P < .05; **P < .01 compared with OVA given alone or with PAR-2CP.

As noted above we observed that mice challenged with OVA and PAR-2AP had a strong mononuclear cell infiltrate. To address what cell type these mononuclear cells were we stained BAL samples with cell specific markers for T cells (CD3), B cells (CD19), DCs (CD11c), and NK cells (DX5). None of these cells were in higher numbers in mice given PAR-2AP with OVA challenge (Fig. 4.3).

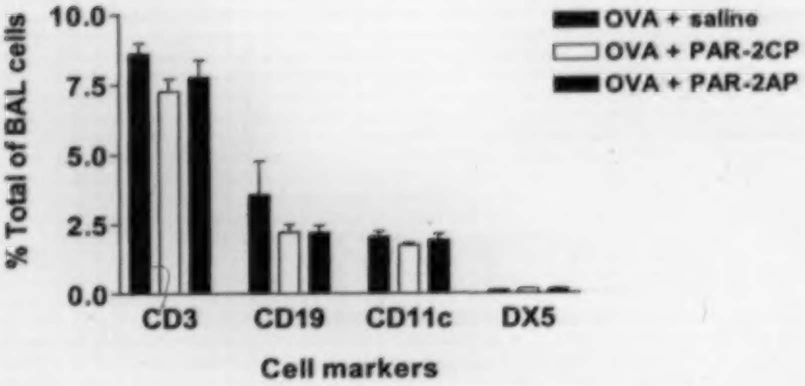

Figure 4.3. Flow cytometric analysis of BAL cells for T cell (CD3; n=9), B cells (CD19; n=6), DCs (CD11c; n=3), and NK cells (DX5; n=3) in mice subject to the short-term protocol (Fig. 4.1A).

To study inflammation occurring in the lung tissue, sections of mouse lungs were prepared from mice sensitized to OVA and challenged according to the short-term protocol (Fig. 4.1A) with OVA + PAR-2CP or OVA + PAR-2AP (Fig. 4.4) and inflammation was observed in a double blinded manner. No differences were seen in the amount of inflammation; however there were differences between the localization of the inflammation. Mice challenged with OVA and PAR-2CP showed inflammatory cuffing around the airways (Fig. 4.4A) as well as the endothelium (Fig. 4.4B). These same mice showed little inflammation in the interstitial regions (Fig. 4.4C). Mice challenged with OVA and PAR-2AP showed less of this cuffing around the airways (Fig. 4.4D) and endothelium (Fig. 4.4E) but showed a more pronounced inflammation in the interstitial area (Fig. 4.4F).

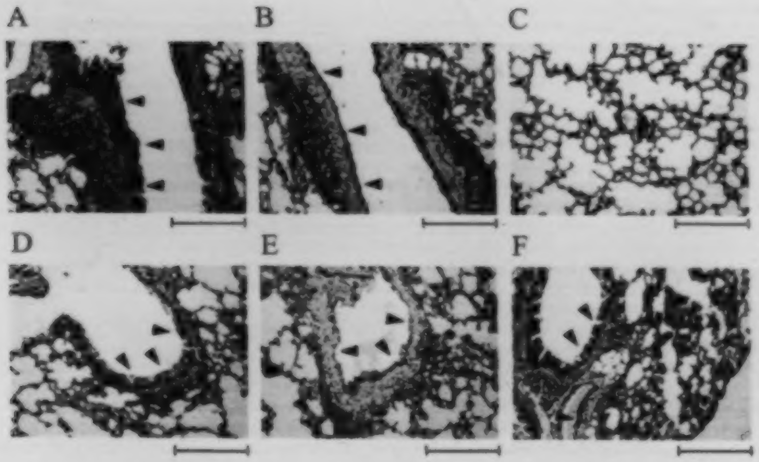

Figure 4.4. Lung inflammation in ovalbumin-sensitized mice challenged with OVA and PAR-2CP (panels A-C) or OVA and PAR-2AP (panels D-F). (A and D) inflammation surrounding the airway epithelium, (B and E) inflammation surrounding the endothelium, and (C and F) inflammation in the interstitial space. Red Arrows indicate the epithelium while Black Arrows indicate the endothelium. The hatched bar represents a 75μm distance. Images are from a single animal challenged with OVA with PAR-2CP or PAR-2AP and are representative of all mice.

A hallmark of murine models of allergic diseases is the production of allergen specific IgE. To determine whether the enhancing effects of the PAR-2AP were IgE dependent, serum levels of OVA-specific IgE were assessed in animals receiving PAR-2AP or PAR-2CP with OVA and in control non-sensitized mice (Table 4.1). We detected OVA-specific IgE in the serum of mice in both treatment groups. PAR-2AP administered with OVA did not increase the levels of OVA-specific IgE when compared to PAR-2CP with OVA.

Table 4.1. Serum levels of OVA-specific IgE in the short-term protocol

Sensitization	*Challenge*	*OVA specific IgE (OD_{405})*
none	*saline*	*0.12 ± 0.05*
OVA + $Al(OH)_3$	*OVA + PAR-2CP*	*0.40 ± 0.04***
OVA + $Al(OH)_3$	*OVA + PAR-2AP*	*0.44 ± 0.03***

*Groups of mice were subject to the short-term protocol and 24h after the final challenge the total circulating volume of blood was taken, serum collected and OVA-specific IgE measured. Results are shown as mean ± SEM. **$p<0.01$ compared to non-sensitized animals challenged with saline. ($n=4$)*

To explore whether the PAR-2-induced enhancement to airway inflammation and AHR were long lasting we evaluated animals 5 days after the final challenge (day19, follow-up protocol seen in Fig. 4.1B). There were no differences in total BAL cell numbers between any of the groups (Fig. 4.2B upper panel) although inflammation as a whole was increased compared to animals examined on day 15. Mice that received OVA with PAR-2AP had $3.3 \pm 0.2\times10^6$ cells in their BAL while mice receiving OVA alone had $3.5 \pm 0.2\times10^6$ cells while mice given OVA with the

$3.4 \pm 0.3 \times 10^6$ cells. There were also no differences in the differential counts of BAL cells between treatment groups. Therefore, the enhanced inflammation seen following PAR-2AP administration dissipated 5 days after the last challenge. Although no differences were seen in inflammation, AHR was still enhanced in the PAR-2AP treated mice. Assessment on day 19 showed that OVA plus PAR-2AP treated mice exhibited a significantly higher MeCh responsiveness when compared to OVA alone or OVA plus PAR-2CP ($p<0.01$) (Fig. 4.2B lower panel). Therefore, the effect of PAR-2 activation on allergen-induced AHR was long lived (≥ 5d).

The above experiment showed that the enhancing effect of PAR-2AP on airway inflammation was lost with time as the inflammation seen in the other treatment groups caught up with time. However asthmatic airways are constantly exposed to proteinases whether they are exogenous or endogenous in nature.. To mimic this chronic PAR-2 activation we administered on days 16 and 18 PAR-2AP, PAR-2CP, or saline to mice that had been challenged on days 12 and 14 with OVA plus PAR-2AP, OVA plus PAR-2CP, or OVA alone respectively. Assessment of inflammation by BAL and AHR by plethysmography followed on day 19 (chronic protocol seen in Fig. 4.1C). PAR-2AP administration in this situation increased total inflammatory cell numbers in the BAL ($5.6 \pm 0.7 \times 10^6$) compared to PAR-2CP ($2.6 \pm 0.3 \times 10^6$) or saline ($2.7 \pm 0.4 \times 10^6$) (Fig. 4.2C upper panel). PAR-2AP treatment increased the numbers of eosinophils ($4.1 \pm 0.5 \times 10^6$) and mononuclear cells ($1.3 \pm 0.2 \times 10^6$) compared to PAR-2CP ($2.2 \pm 0.3 \times 10^6$; $p<0.05$ and $3.2 \pm 0.2 \times 10^5$; $p<0.01$ respectively) or saline ($2.6 \pm 0.1 \times 10^6$; $p<0.05$ and $3.0 \pm 0.6 \times 10^5$; $p<0.01$ respectively). Thus, sustained PAR-2AP administration further enhanced OVA-induced airway inflammation. The difference in AHR seen in the follow-up protocol remained as MeCh responsiveness in PAR-2AP treated mice was still significantly enhanced when compared to the PAR-2CP or saline curves (Fig. 4.2C lower panel).

ii. PAR-2 expression on recruited BAL cells

The PAR-2AP administered intranasally could also affect inflammatory cells recruited during the development of airway inflammation. To explore whether PAR-2AP could affect these cells directly we studied the expression of PAR-2 by these cells. Immunohistochemistry using the B5 Ab showed that inflammatory cells, consisting of eosinophils and mononuclear cells, stained positive for PAR-2 (Fig. 4.5A). Preabsorption with immunizing peptide abolished all staining (Fig. 4.5B).

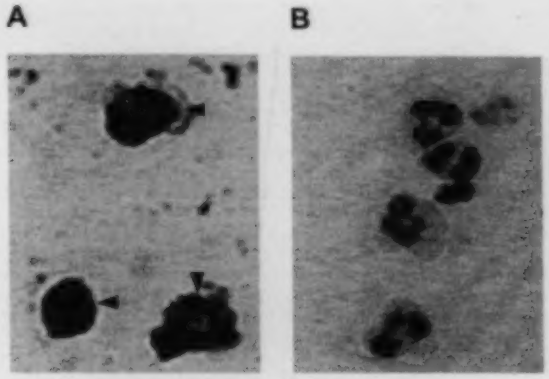

Figure 4.5. PAR-2 localization on inflammatory cells (A). Of the inflammatory cells, PAR-2 was localized to eosinophils (red arrows) and mononuclear cells (black arrow). As a negative control, B5 Ab was preabsorbed with immunizing peptide (B).

ii. Cytokine and chemokine mRNA expression in the lung

To further define the mechanism of enhanced airway inflammation and AHR we studied cytokine/chemokine expression in the lung following one administration of PAR-2AP with OVA, or following the first administration of peptides alone in the chronic protocol. PAR-2AP administered with OVA increased cytokine/chemokine expression in the lung 4 h following challenge. TNF and IL-5 were the most noticeable of these (Fig. 4.6). All differences resolved by 8 h.

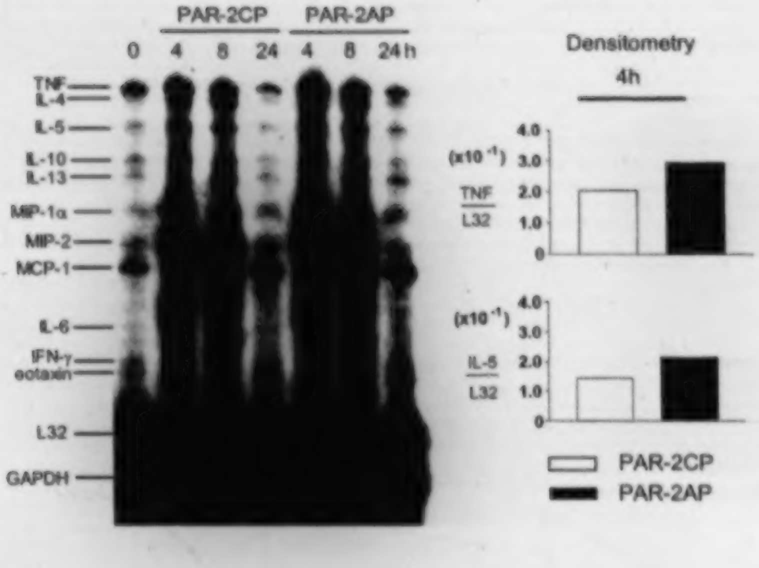

Figure 4.6. Ribonuclease (RNase) protection assay for inflammatory cytokines present in the lung 0, 4, 8, and 24 h after one challenge of OVA with PAR-2CP or PAR-2AP in sensitized mice and densitometry graphs of upregulated genes (1 representative experiment of 2 is shown. RNase protection assay was performed using mRNA isolated from one mouse given OVA with PAR-2CP or PAR-2AP per experiment.

iii. Cytokine levels in BAL fluid

We also looked at cytokine levels in BAL fluid of mice in our protocols. In the Short-term protocol as levels of IL-13 (112.2 ± 92.5 pg/ml vs. undetectable levels, $n = 3$, $p<0.05$) and TNF (81.9 ± 19.2 vs. 9.3 ± 5.8 pg/ml, $n = 3$, $p<0.01$) (Fig. 4.7A) were increased when PAR-2AP was administered with OVA compared to PAR-2CP with OVA. This increase in IL-13 (32.8 ± 26.6 pg/ml vs. undetectable levels, $n = 3$, $p<0.05$) and TNF (100.0 ± 27.1 vs. 18.8 ± 11.9 pg/ml, $n = 3$, $p<0.05$) carried over into the follow-up protocol (Fig. 4.7B). In the Chronic protocol only IL-13 was increased with PAR-2ap treatment (244.7 ± 119.6 vs. 66.3 ± 22.4 pg/ml, $n = 3$, $p<0.05$) while no difference was seen in TNF levels (58.5 ± 27.4 vs. 49.8 ± 25.0 pg/ml, $n = 3$) (Fig. 4.7C). In all cases IL-4, IL-9, IL-10 and IFN-γ were not significantly altered while IL-5 showed an upward trend but did not reach statistical significance.

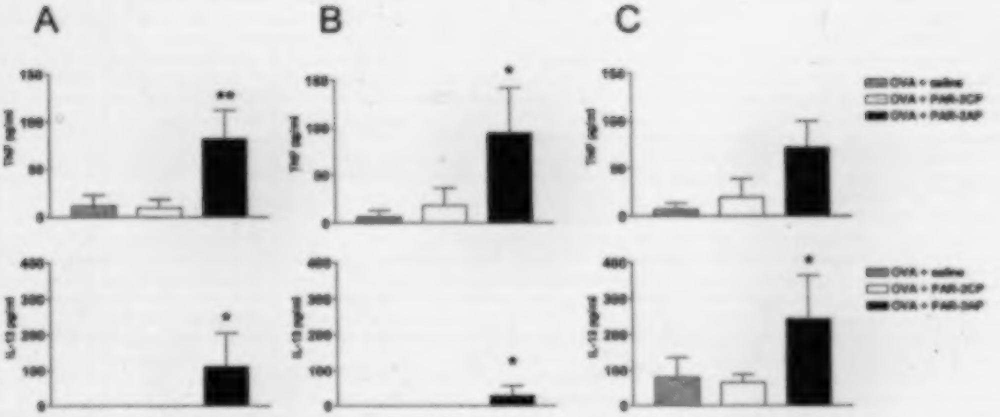

Figure 4.7. TNF (upper panels) and IL-13 (lower panels) levels in BAL fluid of mice with ovalbumin challenge in the (A) short-term, (B) follow-up, or (C) chronic activation protocols as outlined in Fig. 1. Results are shown as means ± SEMs (n = 3). *P < .05; **P < .01 compared with OVA given alone or with PAR-2CP.

IV. Discussion

The literature indicates that PAR-2 activation in the airways may have both protective and pathogenic roles in inflammatory airway diseases depending on the model used and the route of PAR-2 activation (19,26,27). We have shown that administration of a PAR-2AP enhanced OVA-induced airway inflammation and AHR. These results indicate that PAR-2 activation in the airways may have detrimental effects in asthma.

A role for PAR-2 in allergic airway inflammation has been previously shown (17). In that study the authors used PAR-2 knockout mice to study the role of endogenous proteinases, released from inflammatory cells, in allergic inflammation and AHR. In contrast, in our study we challenged normal mice with OVA and PAR-2AP to mimic the effects of inhaled allergens that have the potential to activate PAR-2. In this paper we have shown for the first time that although PAR-2 may be involved in both airway inflammation and AHR, it regulates these two principle features of asthma through different pathways. We have demonstrated that AHR remains elevated for over 5 days following exogenous PAR-2 activation, while the enhancement to airway inflammation was short lived. We have also shown, for the first time, that PAR-2 activation in inflamed airways increases airway inflammation even in the absence of continued allergen exposure. This observation indicates that, in cases of established allergic airway inflammation, non-specific stimuli that activate PAR-2 are sufficient to perpetuate allergic inflammation. These non-specific stimuli may include both exogenous and/or endogenous serine proteinases. This observation may explain why allergen avoidance is not very effective as the sole therapeutic measure as long as airway inflammation persists.

The demonstrated changes in AHR correlated with the increased IL-13 seen in the BAL fluid of PAR-2AP treated mice. This increase in IL-13 could potentiate the responsiveness of airway smooth muscle to methacholine following allergen challenge. Such an effect could then be translated into elevated AHR once inflammation begins. IL-13 has shown to be critical in the development of allergen induced AHR in Balb/c mice, and its function cannot be compensated for by IL-4 nor does it correlate with eosinophil airway inflammation (28). Indeed, it has been demonstrated that administering recombinant IL-13 to the airway of Balb/c mice induces a rapid and potent AHR response before airway inflammation is seen (29). TNF seen in the BAL fluid may be responsible for the enhanced inflammation through increased adhesion molecule expression on the endothelium (30).

Because we administered the PAR-2AP intranasally, it is very likely that its effects were mediated through PAR-2 activation on the epithelium of the upper or lower airways. PAR-2 activation on human airway epithelial cells induces the release of pro-inflammatory mediators such as eotaxin, GM-CSF (11), and matrix-metalloproteinases (12), which are capable of promoting eosinophil recruitment and survival in the lung. Furthermore, these pro-inflammatory mediators released, as result of PAR-2 activation, could be responsible for the differences in localization of the inflammation in the lung. When mice were challenged with OVA and PAR-2CP inflammatory cuffing was seen around the airways and the endothelium. This cuffing was not as pronounced in mice challenged with OVA and PAR-2AP. These animals showed a more interstitial inflammatory localization. The mediators released could be promoting the migration of the cells away from the blood vessels and towards the airways. This could account for the mice given the PAR-2AP to show a more interstitial inflammation as the cells would migrate towards the airways through this space. These mediators could also be responsible for pulling the inflammatory cells into the airways and account for the differences seen in the BAL numbers of mice challenged with OVA and PAR-2AP.

If the effects seen in this study are the result of PAR-2 activation of the epithelium in the nose it would be consistent with the "one airway/one disease" concept (31,32) in which inflammatory events in the nose can promote similar events in the lower airway and vice versa. Release of pro-inflammatory mediators from the airway epithelium following PAR-2 activation could be the cause of increased eosinophilic inflammation in our model.

As shown in chapter 1 PAR-2AP was not capable of initiating airway inflammation of AHR if given in the absence of allergen challenge. This could mean that the PAR-2 agonist is working synergistically with the pro-inflammatory effects mediated by allergen challenge. For example, it may activate PAR-2 expressing inflammatory cells that accumulate in the tissues. We have shown that eosinophils, which predominated in the BAL of OVA challenged mice, express PAR-2. PAR-2-mediated activation of eosinophils induces degranulation and superoxide production (33). Alternatively, it could also mean that the increased epithelial permeability or epithelial desquamation induced by allergen challenge allows the PAR-2AP to penetrate deeper into the lung.

Endothelium throughout the lung also stained positive for PAR-2. PAR-2-mediated endothelial cell activation releases pro-inflammatory cytokines that could affect airway inflammation (34) and increase endothelial permeability (13,35) allowing more inflammatory cell infiltration. Similarly, peripheral nerves have been implicated in PAR-2-mediated neurogenic inflammation (36) and may be of greater access for the PAR-2AP following allergen challenge. Thus, PAR-2 activation of any or all of these cell types may explain further increases in inflammation when PAR-2AP is administered alone following initial challenges.

We propose that allergens possessing intrinsic serine protease activity may be more potent in inducing airway inflammation and AHR because of their PAR-2 mediated effects. Our model also indicates that PAR-2 activation in the airways by inhaled particles or contaminants with serine protease activity may exacerbate established allergen-induced airway inflammation and AHR. The effects of PAR-2 activation occur independently of continuing allergen exposure. Therefore, further exploration of the role of PAR-2 in allergic airway inflammation may offer new targets for therapeutic intervention.

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Chapter 5 – General Discussion and Conclusions

I. Summary of the major findings and their relation to the original aims/hypothesis

Is PAR-2 activation, in the absence of antigen, sufficient to cause the development of AHR and eosinophilic inflammation?

PAR-2 was located by immuno-histological methods using lung sections from sensitized animals and a rabbit polyclonal Ab (B5) that was generated using the N-terminal region of the PAR-2 receptor. We localized PAR-2 to alveolar macrophages, airway epithelium, and smooth muscle in the lungs. To test whether or not PAR-2 could cause development of AHR and eosinophilic airway inflammation we used a Balb/c mouse model system. Mice were administered PAR-2AP alone i.n. that activates PAR-2 directly, thereby bypassing the proteinase/tethered ligand mechanism. In all cases a scrambled version of this PAR-2AP was used as a control.

We observed that although PAR-2AP did not induce pathological features of asthma, AHR or eosinophilic airway inflammation, it induced up-regulation of inflammatory cyokines at both the mRNA and protein levels. TNF and to a smaller extent IL-13, mRNA was upregulated while TNF and IL-13 was expressed at the protein level.

TNF has potential immunogenic properties through its activation of DCs. Potent allergens either possess intrinsic proteinase activity or are inhaled in particles that are rich in serine proteinases. Furthermore, some of the intensely studied allergens can activate PAR-2. We therefore hypothesized that genetically predisposed individuals become sensitized to these allergens following airway mucosal exposure as a result of PAR-2 activation and subsequent release of TNF. This TNF release in turn constitutes a "danger signal" that pushes the immune system into a Th2 response.

Role of PAR-2 activation in the allergic sensitization phase of asthma

We hypothesized that TNF released, following PAR-2 activation, mediates allergic sensitization to inhaled proteins. To test this hypothesis we used a murine system with OVA and PAR-2AP to mimic the potential of a proteolytic allergen, or other inhaled proteinases, to activate PAR-2. Upon OVA re-exposure mice initially administered OVA with the PAR-2AP developed airway inflammation and airway hyperresponsiveness (AHR), produced OVA-specific IgE as well as OVA specific T cells with a Th2 cytokine profile. Conversely, mice given OVA alone or with a control peptide (PAR-2CP) developed tolerance. These tolerant mice did not develop airway inflammation, AHR or OVA-specific IgE, but developed OVA-specific T cells that secreted high levels of IL-10 indicative of $T_{reg}1$ or Th3 function. Furthermore, we confirmed that a TNF mediated pathway is involved as TNF neutralizing Abs given before OVA and PAR-2AP administration, were able to prevent sensitization.

Role of PAR-2 activation in the effector phase of asthma

After showing that TNF release was important in the development of allergic sensitization we focused on the importance of PAR-2 activation in the airways during allergen challenge (effector phase of asthma). We hypothesized that heightened PAR-2 activation in the airways during allergen challenge would exacerbate the pathological conditions of asthma by the release of greater amounts of inflammatory mediators such as TNF. To study this hypothesis PAR-2AP or PAR-2CP was administered with OVA i.n. to mice that had been previously sensitized to OVA. AHR and eosinophilic airway inflammation were evaluated. Mice challenged with OVA alone, or challenged with OVA and PAR-2CP, developed comparable amounts of AHR and eosinophilic airway inflammation. When PAR-2AP was administered during OVA challenge AHR and airway inflammation were enhanced. When evaluated 5 days later the enhanced AHR persisted, while the enhancement to airway inflammation dissipated. Mice administered PAR-2AP alone during the 5 days following the final antigen challenge exhibited enhanced airway inflammation when compared to the control animals, but did not show increased AHR. This led us to propose that the mechanisms by which the PAR-2AP mediates its effects on airway inflammation and AHR differ. We also showed that PAR-2AP administered with allergen increased TNF and IL-5 mRNA in lung tissue and IL-13 and TNF in BAL fluid. This up-regulation of mediators may be the mechanism by which enhanced PAR-2 activation was mediates its effects.

II. Conceptual model Revisited

Our original conceptual models focused around PAR-2 activation in the airways promoting release of mediators that facilitate atopic/allergic sensitization or exacerbate asthmatic conditions during the effector phase of the allergic response. In chapter 2 we found that when PAR-2AP was administered into the airways alone, TNF levels were increased at both the mRNA and protein levels. As mentioned TNF is of particular interest as it is not only capable of initiating immune responses but also is integral in the inflammatory response during the effector phase of type I hypersensitivity reactions such as asthma. Because of this we based the following conceptual models around TNF release following PAR-2 activation.

Conceptual model: PAR-2 activation promotes allergic sensitization to OVA through a TNF mediated pathway

In chapter 3 we showed that OVA administered i.n. alone, or with PAR-2CP, promoted mucosal immune tolerance that protected the animals against the development of allergic inflammation. However, when OVA was given with PAR-2AP mice developed allergic inflammation in the lung. We hypothesized that TNF released following PAR-2 activation was important in the sensitization process. Indeed, in chapter 3 we showed that when a neutralizing Ab to TNF was administered, prior to OVA and PAR-2AP administrations, allergic sensitization to the OVA did not occur. As the PAR-2AP was administered i.n. it is likely that AMs and the epithelium are the cell types targeted by the PAR-2AP. Therefore, we hypothesize that PAR-2 activation of AM or the epithelium, or both concurrently, promotes the release of TNF that acts as the danger signal that pushes the immune response towards allergic sensitization (Fig. 5.1).

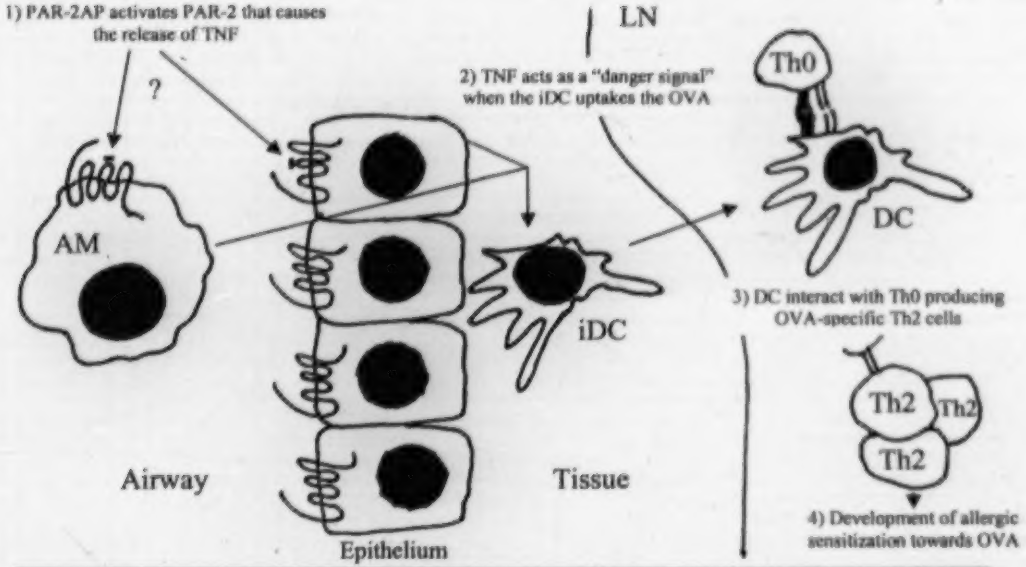
1) PAR-2AP activates PAR-2 that causes the release of TNF
?
LN
2) TNF acts as a "danger signal" when the iDC uptakes the OVA
Th0
DC
AM
iDC
3) DC interact with Th0 producing OVA-specific Th2 cells
Th2
Th2
Th2
Airway
Tissue
4) Development of allergic sensitization towards OVA
Epithelium

Figure 5.1. PAR-2 activation interferes with the development of mucosal immune tolerance and promotes allergic sensitization through a TNF mediated pathway. AM = alveolar macrophage, iDC = immature DC, Th0 = naïve T cell, Th2 = T-helper-2 cell.

Conceptual model: PAR-2 activation in the airways during allergen challenge exacerbates the pathological features of asthma

In chapter 4 we showed that when OVA sensitized mice were challenged twice with OVA alone, or concurrently PAR-2CP, they developed eosinophilic airway inflammation and AHR. Cellular inflammation in the lung consisted of eosinophils and neutrophils. This lung inflammation showed a cuffing pattern around the airway epithelium as well as the endothelium (Fig. 5.2). However when mice were challenged with OVA along with the PAR-2AP eosinophilic airway inflammation and AHR were enhanced. Moreover, there was a strong mononuclear airway infiltration. Cellular inflammation in the lung showed a more interstitial pattern. We propose that when administered into the airways concurrently with OVA, PAR-2AP activates PAR-2 on cells in the airway (#1 in Fig. 5.3). causing the release of inflammatory cytokines and chemokines. This results in the migration of the eosinophils cuffed underneath the epithelium and surrounding the endothelium into the airways and interstitial space respectively (#2 in Fig. 5.3). Concurrently, mononuclear cell (Mo) infiltration into the airways is greatly enhanced. We also propose that these inflammatory mediators are directly or indirectly responsible for the enhancement of AHR.

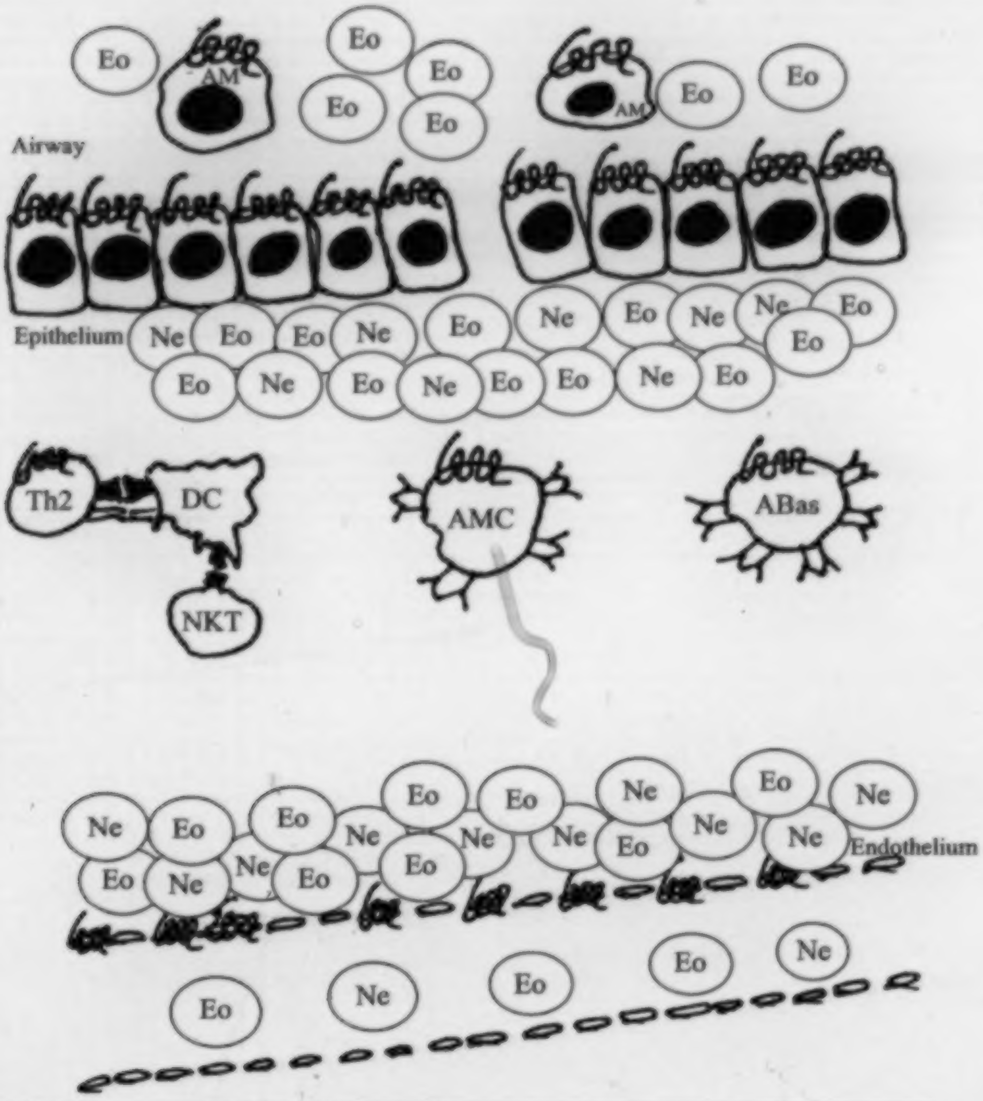

Figure 5.2. OVA sensitized mice challenged with OVA with PAR-2CP show a cuffing of eosinophils and neutrophils around the airway epithelium and endothelium. AM = alveolar macrophage, Eo = eosinophil, Ne = neutrophil, Th2 = T-helper-2 cell, AMC = activated mast cell, ABas = activated basophil.

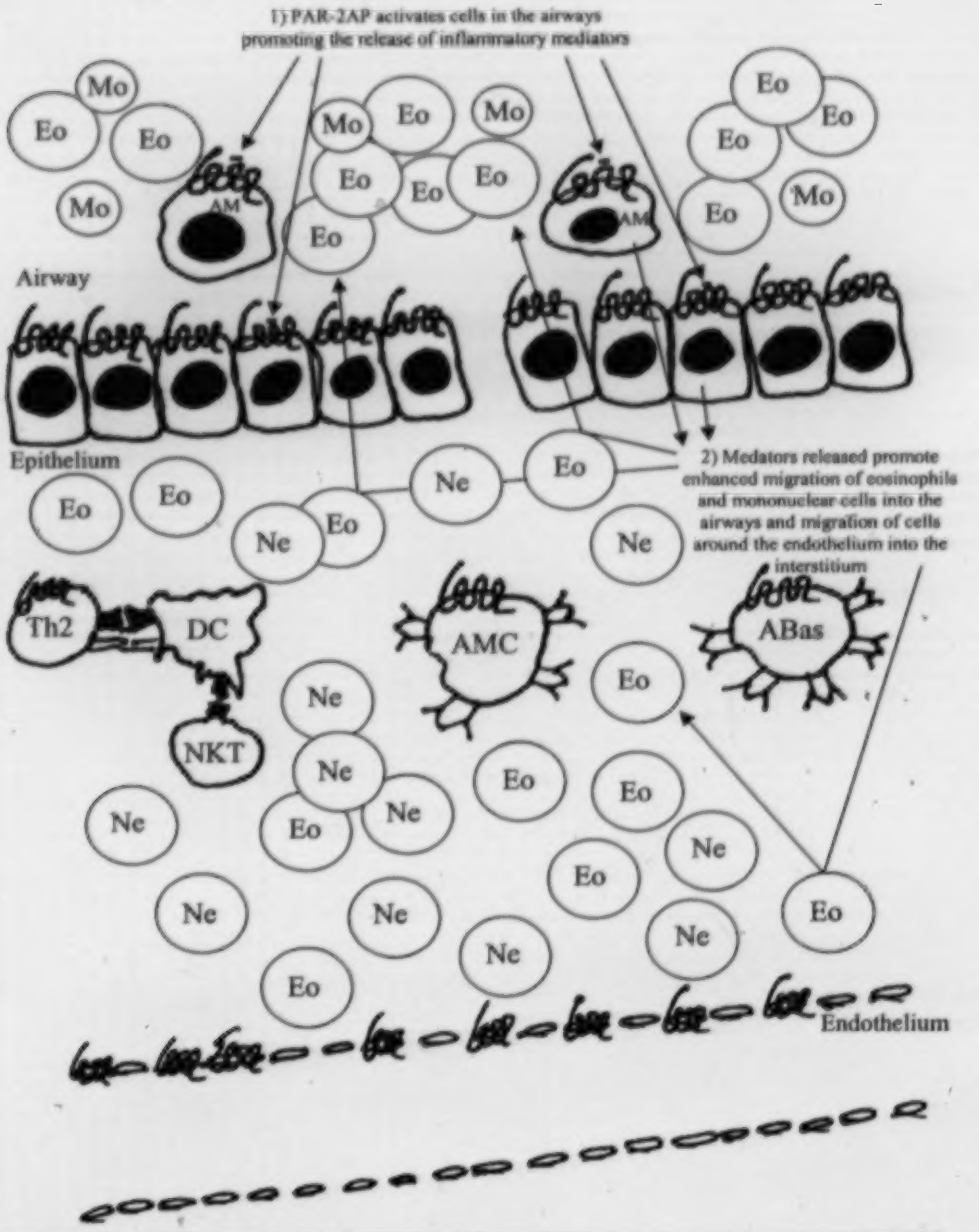
1) PAR-2AP activates cells in the airways promoting the release of inflammatory mediators
Mo
Eo
AM
Airway
Epithelium
2) Medators released promote enhanced migration of eosinophils and mononuclear cells into the airways and migration of cells around the endothelium into the interstitium
Ne
Th2
DC
NKT
AMC
ABas
Endothelium

Figure 5.3. Conceptual model of the effects of enhanced PAR-2 activation during OVA challenge. AM = alveolar macrophage, Eo = eosinophil, Ne = neutrophil, Th2 = T-helper-2 cell, AMC = activated mast cell, ABas = activated basophil, Mo = mononuclear cell

III. Results and their relation to the current literature

Major finding #1: PAR-2 activation promotes allergic sensitization to OVA through a TNF mediated pathway

In chapter 3 we showed that PAR-2 activation in the airways was able to promote the development of allergic sensitization, and the subsequent development of allergic inflammation in the airways, towards OVA antigen. As discussed in chapters 1 and chapter 3, OVA normally causes the development of mucosal immune tolerance that acts as a protective mechanism against the development of disease following mucosal exposure. These protective effects are mediated by antigen-specific $T_{reg}1$ or Th3 cells that are able to prevent the development of antigen-specific Th2 cells by the release of regulatory mediators like IL-10.

Our results are consistent with the Devlin *et al* study showing that PAR-2 activation as a key factor in the allergic response post infection in mice (1). Many parasitic helminths provoke a Th2/ allergic immune response characterized by elevated expression of IL-4, IL-10 and/or high levels of IgE (2). Why these parasites induce a Th2 response towards them is not fully understood. However, parasites often use proteinases to assist in invasion and establishment in the host (3). Devlin *et al* compared the infectivity of the nematode *N. brasiliensis* in mice lacking the PAR-2 gene and their wild-type littermates. They found that PAR-2KO mice displayed elevated fecal egg counts and decreased levels of total serum IgE following subcutaneous infection with third-stage *N. brasiliensis* larvae. To further support their work the authors gave mice OVA along with a PAR-2AP subcutaneous, in the same manner as the *N. brasiliensis* larvae, to address whether PAR-2 activation would facilitates a Th2 response towards OVA. They found that when given in this manner the immunological hallmarks of parasite infection, IgE- and IL-10-expressing lymphocytes, were present in mice given OVA with PAR-2AP, but not in those given PAR-2CP. Their results show that not only is PAR-2 of importance in *N. brasiliensis* infection, but it also shows the ability of PAR-2 to promote a Th2 response towards the innocuous antigen OVA.

Both our study and the Devlin *et al* study suggests that PAR-2 acts as a proteinase receptor that can induce an allergic immune response. The differences between the study in chapter 3 and the Devlin *et al N. brasiliensis* study is that we identified TNF as playing a role in sensitization.

Our study also has similarities to one conducted by Fattouh *et al* showing allergic sensitization towards OVA when administered into the airways of mice with HDM extract (4). This sensitization was characterized by an eosinophilic inflammatory response and AHR. Furthermore, these authors observed elevated serum levels of OVA-specific IgE and increased production of Th2 cytokines when splenocytes were stimulated *in vitro* with OVA. These authors showed that GM-CSF was responsible for this response.

As HDM can activate PAR-2, the effects seen by Fattouh *et al* could be the result of the same PAR-2 mediated pathway we identified. Although these authors showed that GM-CSF is important in this sensitization, it does not contradict our study. HDM, through PAR-2 activation, could promote the release of TNF. This TNF could then induce GM-CSF release. This GM-CSF in turn could promote sensitization. Therefore, Fattouh *et al* could have identified an important mediator downstream of the TNF.

Major finding #2: Enhanced PAR-2 activation in the airways during allergen challenge exacerbates the pathological features of asthma

Our results of a pathological role for PAR-2 in allergic lung disease are consistent with the report of Schmidlin *et al* (5). These authors used a transgenic mouse that over-expressed PAR-2 in the airways. The mice were sensitized and challenged with OVA and the authors saw that both airway inflammation and AHR were greater than in mice with normal expression of PAR-2. Although different in designs this study and ours show that if PAR-2 activation is enhanced then it promotes an increase in the pathogenesis of allergic airway inflammation. The Schmidlin *et al* report does however, differ from our data in that when the PAR-2AP (SLIGRL-NH_2) was administered alone into the airways of the FVB strain of mice an infiltration of macrophages was seen by BAL. We found no such infiltration when we gave the same PAR-2AP alone to Balb/c mice. Interestingly this appears to indicate differential effects of PAR-2 activation between the two mouse strains.

Although our results are consistent with those of Schmidlin *et al*, they are contradictory with a recent report by D'Agostino *et al* showing that PAR-2 activation reduces the levels of AHR and airway inflammation (6). D'Agostino *et al* used a rabbit model of allergic asthma where the animals were sensitized and challenged with allergenic extracts from the pollen of the *P. judaica* weed. During challenge with the extract rabbits were given either a PAR-2AP (SLIGRL-NH_2) or PAR-2CP (LSIGRL-NH_2). The authors showed that PAR-2 activation provided a protective role in the airways by reducing the pathological features of asthma. The control peptide had no effects. There are some differences that could explain the opposing effects of PAR-2 activation during allergen challenge in this work and ours. The most important of these is the dose of PAR-2AP given. D'Agostino *et al* used PAR-2AP at a dose of 10 mg/kg, while in our study animals were given 8.1 µg/kg. Perhaps different doses of PAR-2AP have different effects on the levels of activation. For instance at lower levels of PAR-2 activation, as one would expect from the endogenous proteinases released in the lungs during allergen challenge, PAR-2, may promote the development of the pathological features of the disease. At more moderate levels of PAR-2 activation, as seen in our studies using lower doses of PAR-2AP and in the Schmidlin *et al* study, this enhanced activation in the lung leads to an exacerbation of the disease and thus the levels of AHR and airway inflammation worsen. However, at high levels of PAR-2 activation, as seen in D'Agostino *et al* study, PAR-2 may have protective roles in the airways. Different levels of PAR-2 activation may promote the release of different mediators which in turn could have pro or anti-inflammatory results. The differences seen in the D'Agostino *et al* study could be the result of the PAR-2AP penetrating further into the lung and activating cells that a lower dose would be incapable of.

High doses of PAR-2AP have also been shown to be anti-inflammatory in a mouse model of LPS-induced neutrophilic pulmonary inflammation (7). Moffatt *et al* showed that LPS alone in Balb/c mice induced a strong neutrophilic pulmonary inflammation. However, this inflammation was markedly decreased in animals receiving a high dose of PAR-2AP (approx. 25 mg/kg of SLIGRL-NH_2) with the

LPS. This supports the possibility of different physiological responses occurring depending on the level of PAR-2 activation. However, one may question how physiological such high levels of PAR-2 activation actually is and whether or not such a level could ever be obtained in asthma or any disease state involving PAR-2.

IV. Relevance and clinical significance of our findings

Major finding #1: PAR-2 activation promotes allergic sensitization to OVA through a TNF mediated pathway

This study has shown that receptors other than TLRs are able to initiate specific immune responses once activated. Our study is the first to show a mechanism by which PAR-2 activation promotes atopic/allergic sensitization towards an innocuous protein that normally promotes mucosal immune tolerance. This mechanism involves the release of TNF once PAR-2 is activated. It is conceivable that the PAR-2 activating potential of many potent allergens harboring proteinase activity mediates allergic sensitization. Thus interfering with their activation of PAR-2 could be protective by promoting the development of mucosal tolerance. This allergen specific tolerance then could then protect the individual from subsequent allergic sensitization to the proteolytic allergen.

Major finding #2: Enhanced PAR-2 activation in the airways during allergen challenge exacerbates the pathological features of asthma.

This study supports the results of Schmidlin *et al* and the hypothesis that PAR-2 activation is integral in the pathology of allergic asthma. On the other hand it conflicts with the D'Agostino *et al* study showing that PAR-2 activation can be protective. Looking at are results together, it is possible that different approaches could be used to treat asthma by targeting PAR-2. On one hand you could interfere with the activation of PAR-2 by the endogenous proteinases released in the lungs during allergen challenge. This could alter release of mediators, such as TNF, and could lead to a reduction of the pathology of the disease. On the other hand one could target PAR-2 by activating it throughout the airways. This could lead to the release of the anti-inflammatory mediators which in turn could lead to a reduction of AHR and airway inflammation.

V. Future directions

Major finding #1: PAR-2 activation promotes allergic sensitization to OVA through a TNF mediated pathway

When we administered OVA in the airways with PAR-2AP we altered the immune system towards atopic/allergic sensitization and away from mucosal tolerance. Although TNF was integral in this process, the exact mechanisms by which PAR-2 acts is unknown. To define the precise mechanism, we would have to define what cell type/s PAR-2 activates. Since OVA and PAR-2AP were given to the animals i.n., we hypothesize that PAR-2AP mediates its initial effects on either AM or the airway epithelium, as these are the first cell types that PAR-2AP would encounter.

PAR-2 activation on AM being the primary mechanism

As we showed in chapter 2 AM have high expression of PAR-2. To determine whether or not PAR-2 on AM is relavant in our model, AM could be depleted using liposomes composed of phosphatidylcholine and cholesterol (6:1 molar ratio) and containing dichloro-methylene-diphosphate (DMDP) in saline. This treatment, when given locally in the lung, selectively depletes AM and does not cause influx of inflammatory cells (8). Such treatments eliminate AM from the airways for more than a week (8-11). Following the elimination of AM with DMDP- containing liposomes, these mice would be administered AM isolated from PAR-2KO mice. This is vital as the AM could be important in generating tolerogenic signals in the airways. Using this system when we give the OVA with the PAR-2AP and tolerance towards the OVA develops then it is likely that the PAR-2AP is mediating its effects on PAR-2 on the AM. Furthermore, if we could determine that TNF is released when activated through their PAR-2 receptor then a mechanism on how PAR-2 in the airways is promoting the development of allergic sensitization would be known. However, as mentioned above it is that PAR-2 on airway epithelium could be the important factor involved.

PAR-2 activation on the epithelium being the primary mechanism

To address this possibility a different approach would be used. We could create a chimeric mouse where the bone marrow from wild-type mice would be transferred into PAR-2 KO animals. In this case all of the hematopoietic cells would express PAR-2, while structural cells such as epithelial cells would be PAR-2 deficient. If we give OVA with PAR-2AP to such chimeric animals and tolerance towards the OVA does not develop, it is likely that the PAR-2AP is mediating its effects on PAR-2 on the epithelium. As in the case with the AM, determining if the epithelium releases TNF following PAR-2 activation would give us a precise mechanism.

Although I have singled out the AM and epithelium, it is possible that PAR-2 activation on both of these cell types is important in sensitization. It is also possible that it is an entirely different cell type involved in the process. Nonetheless, it would be valuable to know the precise mechanisms involved, as we could determine if the same mechanisms are involved in the sensitization to clinically relevant allergens, such as HDM allergens.

Major finding #2: PAR-2 activation in the airways during allergen challenge exacerbates the pathological features of asthma.

Identify the pathway through which PAR-2 is mediating its effects

Using some of the techniques outlined to determine the mechanism of PAR-2 mediated allergic sensitization, we could investigate the precise cell type/s involved in PAR-2AP effects on inflammation and AHR following OVA challenge. Endogenous proteinases released during allergen challenge could activate PAR-2 on several cell types in the lung that the PAR-2AP in our experiments would not easily access, because PAR-2AP would be unlikely to penetrate into the lung to activate such cells. If endogenous proteinases are important in the pathogenesis in the lung through activation of PAR-2, then interfering with this activation could reduce the level of pathology in the lung. Thus, one could mistakenly think that this was the mechanism by which the PAR-2AP was mediating its effects when in fact this was not.

Identify the mononuclear cell infiltrate

When the PAR-2AP was given during allergen challenge there was a significant mononuclear infiltrate in the BAL along with the eosinophils. Through flow staining of BAL cells we determined that these cells were not T cells, B cells, dendritic cells, or NK cells. They could however be NKT cells or another type of mononuclear cell. Determining what these cells are it could provide insight into how PAR-2AP has its effects. For instance if the cell type/s is/are determined then one could search what cells have shown to produce and release chemoattractants for that cell type. Using this information one could pinpoint how PAR-2AP mediates its effects.

Identify whether different levels of PAR-2 activation in the airways produces different inflammatory results during allergen challenge

Earlier in this discussion I mentioned that the literature appears to be divided as to whether or not PAR-2 activation enhances the pathology of experimental asthma or whether it is anti-inflammatory and thus protective. As mentioned these differing results could be due to the amounts of PAR-2AP given to the animals during challenge. Perhaps low to moderate levels of PAR-2 activation are pathogenic, while high levels of PAR-2 activation are protective. It would be interesting to determine if this range of PAR-2 activation does determine pathogenic or protective effects and what the mechanisms could be in each of the cases.

Knowing the mechanisms involved in PAR-2 mediated allergic sensitization and enhancement to allergic lung inflammation can give us more insight into the roles this receptor plays in initiating and promoting allergic disease. This could open up new therapeutic targets that could be used in treatment of allergic diseases such as asthma.

VI. References

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